JOURNAL OF THE NATIONAL CANCER INSTITUTE

National Institutes of Health • Public Health Service

Volume 26 • Number 2

February 1961

JOURNAL OF THE
NATIONAL
CANCER
INSTITUTE

Long-Term *In Vitro* Cultivation of Some Mouse Ascites Tumors: Ehrlich Ascites Carcinoma [1, 2, 3]

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SUMMARY

Ehrlich ascites tumor cells have been grown for more than a year in tissue culture. Changes in morphology, cell size, and chromosome number occurred with variations in the media used. Cells grown on fetal calf serum were predominantly small and spindle-shaped with a chromosome number ranging around a median of 65. Cells grown on ascitic fluid medium and later cloned were frequently multinucleate, ameboid in shape, larger, and often contained a higher number of chromosomes. Tissue-culture strains of cells, when injected at a level of 10^5 cells or higher, produced characteristic ascites tumors in mice even after 36 transfers and 9 months' growth *in vitro*. Doses of 10^3 cells in general produced very few tumors (less than 20%) and apparently induced a high degree of resistance against a subsequent lethal dose of 10^6 tissue-culture strain cells. Protection against the mouse-grown Ehrlich ascites cells was less marked, but a significant increase in survival time occurred.—J. Nat. Cancer Inst. 26: 271–281, 1961.

A CONSIDERABLE amount of literature has accumulated on the subject of ascites tumors in general, as two volumes of the Annals of the New York Academy of Science which appeared in 1956 (*1*) and 1958 (*2*) clearly demonstrate. However, the number of ascites tumors established in tissue culture is relatively low, and has been studied actively only during the past 3 years. A characteristic but incomplete list is mentioned here (*3–8*).

Considering the many metabolic studies which have been conducted with Ehrlich ascites tumor cells, we thought the establishment of a tissue-culture strain would be highly useful. At the time this cell line was isolated, June 2, 1959, no other strains were known to exist, as judged from the available literature. While Ehrlich ascites carcinoma had been grown by Hull (*3*) as early as 1953, the strain was accidentally lost and never re-

[1] Received for publication July 11, 1960.

[2] This investigation was supported in part by grant L-37 from the American Cancer Society, Inc., and in part by Cancer Research Funds, University of California, San Francisco, Calif.

[3] Part of this work was presented in preliminary form at the meeting of the Tissue Culture Association, Chicago, Ill., April 7, 1960.

[4] I express my appreciation to Dr. David A. Wood, Director of the Cancer Research Institute, University of California Medical Center, for his encouragement and support during this investigation.

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established. Partially successful cultures or maintenance was mentioned by Eaton and Scala (*9*), Graetz *et al.* (*10*), Powell (*11*), and Graff and McCarty (*12*). Early in 1960 Deschner and Allen (*13*) reported on their "established" (80-day) cell line. Recent reports by Guerin and Morgan (*14*) and Foley *et al.* (*15*) have revealed that these workers have had cultures growing for over 1 and 3 years, respectively. While this manuscript was being written, the report of Ely and Gray (*16*) was published and the manuscript of Jackson, Giuffre, and Perlman (*17*) was brought to our attention.

MATERIALS AND METHODS

Source of tumor.—The ES hypotetraploid line of Klein was our source of Ehrlich ascites tumor. It was sent to Dr. David Greenberg of the Department of Biochemistry by Dr. K. Suguira of Sloan-Kettering Institute and has been carried continuously in Swiss mice for over 6 years. During this time the tumor material has been maintained by weekly transfer and has neither been frozen nor cloned. The Ehrlich ascites carcinoma cells were obtained aseptically from the ascitic fluid of 1 of these mice.

Isolation and media.—About 2 ml of the fluid containing a heavy suspension of cells was placed in a 15 ml centrifuge tube with 5 ml of Tyrode's solution. The cells were centrifuged lightly, and the supernatant liquid and top layer of the precipitate containing some red cells were removed. Approximately 2 ml of fetal calf-serum medium was added to the packed ascites cells and mixed. The resulting heavy suspension was inoculated into one T-30 and one T-60 flask and diluted with sterile media. Since, after about 15 minutes, a nearly solid sheet of cells settled on the glass, the medium containing excess floating cells was decanted and fresh medium added.

At intervals of 2 to 4 days, as the *p*H became acid, the used medium and any detached cells were removed and fresh medium was added. Two types of media proved satisfactory: 12 to 15 percent fetal calf serum, 70 percent solution 199, and the remainder 15 to 18 percent Tyrode's salt solution (A); 40 percent human ascitic fluid, 5 percent fetal calf serum, 0.25 percent lactalbumin hydrolysate and solution 199 to volume (B). All media contained antibiotics: penicillin at a level of 50 units and streptomycin at a concentration of 50 μg per ml.

Sufficient growth occurred so that subcultures could be made after 3 weeks and at approximately 1- to 2-week intervals thereafter. During the first few weeks, dilutions varied from 1:3 to 1:10, whereas the more recent subcultures could be made at 1:20 to 1:40 at 5- to 10-day intervals.

Cell size distribution.—When growth was sufficient for subculture, the cells were scraped off the glass, transferred in their medium to a 15 ml centrifuge tube, and mixed with 0.1 to 0.2 ml heparin sodium (equivalent to approximately 1 mg heparin). The cells were then centrifuged lightly, the supernatant decanted, and the packed cells suspended in 0.85 percent NaCl, transferred to a 50 ml graduated cylinder, and diluted to volume.

Further dilution was made if needed to obtain a count of 10,000 to 40,000 per 0.5 ml liquid on the Coulter counter Model A—at a threshold reading of 10. Settings of 6 for the sensitivity and 2 for the aperture were used. Threshold values from 10 to 80 corresponded to cell diameters of 6.6 to 13.2 μ.

Chromosome counts.—The technique of Hsu and Klatt (*18*) was used, with only a few minor modifications. Two ml of a cell suspension was transferred to a 15 ml centrifuge tube and incubated 2 to 3 days in an inclined position. Then 0.1 ml of colchicine at a concentration of 1×10^{-5} was added and further incubation lasted 14 to 18 hours. The tube was then centrifuged, the supernatant decanted, and the cells treated with a solution of 0.1 percent trypsin or 0.05 percent Versene in hypotonic saline (3 parts Dulbecco's saline and 7 parts distilled water) for 15 to 30 minutes, with occasional shaking. The tube was centrifuged lightly, the liquid decanted and drained to leave only a small drop, then a few drops of acetic alcohol were added slowly with constant stirring to avoid clumping. A drop from this fixed preparation was placed on a slide and diluted wet with equal parts aceto-orcein or allowed to dry before the addition of the stain. The preparation was then squashed and mounted.

Median chromosome values were calculated from accurate counts of 50 or more cells. Approximate counts of 200 or more cells were used to calculate the proportion of cells falling into the triploid, tetraploid, hexaploid, etc., categories.

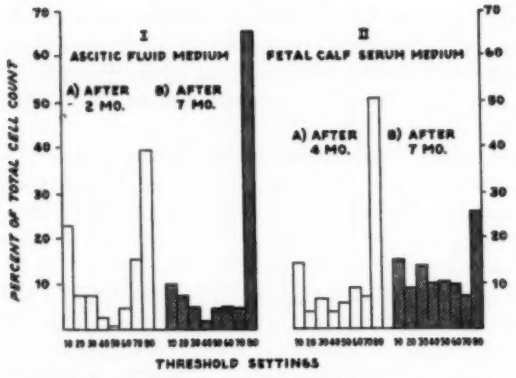
RESULTS

The Ehrlich ascites tumor cells, originally uncloned when obtained from the mouse, showed a considerable range in size. Morphological variations appeared as the tissue-culture lines became established in the two media used. Primarily smaller, slender, spindle-shaped cells (fig. 1) predominated in the fetal calf-serum medium (A), whereas the cells in the ascitic fluid medium (B) were larger and more ameboid in appearance. Cloning experiments made after 9 months' culture in medium B gave rise to 2 sublines similar in appearance. These lines are characterized by the preponderance of the large ameboid cells which are frequently multinucleate (fig. 2).

At various times throughout the culture of these cells *in vitro*, cell-size distribution curves were plotted, with the use of the Coulter counter. Results showed that, as the cells became established in their respective media, the pattern of morphological differences correlated with variations in cell size and chromosome counts. Text-figure 1 illustrates the changes in cell size occurring during 7 months' growth in each medium.

Chromosome Counts

Chromosome counts were carried out at frequent intervals on cultures from both media and also on fresh mouse ascites tumor material. Both the fresh mouse cells and those grown on fetal calf medium showed a



TEXT-FIGURE 1.—Cell-size distribution of Ehrlich ascites tumor cells.

wide range in their chromosome numbers, but the majority fell between 54 to 71 with a mode of 64 or 65 (fig. 3). The range of the majority of cells on medium B in the uncloned cultures was somewhat higher, from 61 to 78 with a mode of 68, though a fair number of cells had counts of 110 to 130 chromosomes. The cloned line on medium B contained fewer cells in the lower range (69–80), whereas a large portion of cells contained between 110 to 130 chromosomes (fig. 4) and some cells contained as many as 160 or more.

Mouse Reinoculation Studies

In all the experiments reported here the tissue-culture strain growing in fetal calf-serum medium was used, as these cells closely resembled the parental mouse strain in both chromosome number and cell-size distribution.

The abbreviations used in relation to the mouse passage and tissue-culture strains reported and depicted in table 1 and text-figure 2 are arbitrarily defined as follows:

MA—mouse-grown cells obtained from routine serial passage of Ehrlich ascites tumor in the Swiss mouse.

TC—tissue-cultured cells obtained from various subcultures on fetal calf-serum medium.

TCS_1—first mouse passage of TC cells.

TCS_2—second mouse passage from TCS_1 cells.

A series of 4 experiments comparing the relative virulence of cells grown in tissue culture with those propagated in mice is summarized in table 1 and shows the following trends:

1) The MA controls demonstrate the high degree of virulence of the parental mouse strain at all levels of dilution used. A dose of 10 cells produces 100 percent mortality with a median survival time of only 21 days.

2) The lowest level of TC cells at which some ascites tumors appear (approximately 25% of the animals tested) is 10^3 cells.

TABLE 1.—Relative virulence of mouse passage (MA) and tissue culture (TC) strains of Ehrlich ascites tumor cells

	Controls MA cells			A = 12th subculture TCS_1 = 8 days TCS_2			B = 17th subculture TCS_1 = 8 days TCS_2			C = 23d subculture TCS_1 = 12 days TCS_2			D = 27th subculture TCS_1		
Number of cells inoculated	Number of mice	MST* (range)	Number dead	Number of mice	MST (range)	Number dead	Number of mice	MST (range)	Number dead	Number of mice	MST (range)	Number dead	Number of mice	MST (range)	Number dead
10^7							2	24 days 23–25	2/2	5	14 days 10–16	5/5			
10^6							5	26 days 20–∞	4/5	5	21 days 19–23	5/5	5	18 days 16–∞	3/5
10^5	10	16 days (10–19)	10/10	10	39 days 23–∞	7/10	5	31 days 19–∞	4/5	5	24 days 22–∞	4/5	5	> 71 days 21–∞	1/5
10^4	10	18 days (14–22)	10/10	10	37–∞	2/10	5	40 days 25–52	5/5	10	> 95 days 27–∞	5/10	5	> 71 days 54–∞	2/5
10^3	10	21.5 days (17–24)	10/10	10	38–∞	1/10	5	> 78 days 47–∞	2/5	5	> 55 days 50–∞	4/5	5	∞	0/5
10^2	10	19.5 days (13–23)	10/10	10	∞	0/10									
10	10	21 days (17–28)	10/10	10	∞	0/10									

*Median survival time.

3) Relative virulence at different dose levels varies from one tissue-culture passage to another. The lowest virulence seems to occur with TC cells in the TCS_1 mice and the highest virulence in the TCS_2 mice inoculated with TCS_1 cells which had remained 12 days in the first passage mice.

4) The lowest level of tissue-culture derived cells (whether TC or TCS_1) capable of averaging better than 50 percent mortality in the Swiss mice is 10^5 cells.

On the basis of these findings, a more comprehensive experiment was devised to test both the virulence and possible immunological properties of the tissue-culture strain (text-fig. 2).

Two groups of 25 mice were immunized as follows:

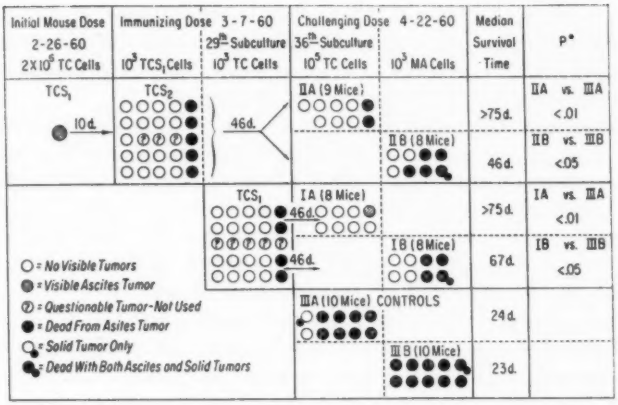
I) Called TCS_1, immunized with 10^3 TC cells from the 29th subculture *in vitro*.

II) Called TCS_2, immunized with TCS_1 cells obtained from an ascites tumor produced in mice inoculated 10 days previously with a massive dose of 2×10^6 TC cells from the 28th subculture.

After we observed these mice for 46 days, during which time 9 mice died with ascites tumors and some appeared doubtful and were discarded, the remaining apparently normal mice showing no visible evidence of ascites tumors were divided and treated as follows:

Group I into two groups:	
IA—inoculated with 10^5 TC cells	(8 mice)
IB—inoculated with 10^3 MA cells	(8 mice)
Group II into two similar groups:	
IIA—inoculated with 10^5 TC cells	(9 mice)
IIB—inoculated with 10^3 MA cells	(8 mice)
Control groups of stock animals included to check the virulence of the TC and MA cells used:	
IIIA—inoculated with 10^5 TC cells	(10 mice)
IIIB—inoculated with 10^3 MA cells	(10 mice)

Results show quite clearly that the MA cells, at a level of 10^3, exhibited their characteristic virulence when inoculated into the unprotected stock animals (group IIIB) and killed 9 of 10 mice within 30 days, while the surviving mouse developed a large solid tumor somewhat later and died after 72 days. The TC cells at a level of 10^5 (a dose 100 times more concentrated than the MA cells) gave quite similar results and killed 80 percent of the mice in 37 days. At 75 days, when the experiment was terminated, 1 mouse appeared normal and the other remaining mouse showed a solid tumor (group IIIA). The mice in groups IA and IIA protected with first or second passage cells grown in tissue culture exhibited a high degree of immunity to the challenging dose of 10^5 TC cells. No deaths occurred in group IA during the 75-day test period, and only 1 mouse showed any visible signs of ascites tumor. In group IIA, 2 mice died with ascites tumors (at 26 and 70 days), but the remaining animals were normal. A lower but significant degree of immunity



TEXT-FIGURE 2.—Protective effects of Ehrlich ascites tumor cells from tissue culture on mice inoculated with lethal doses of mouse passage (MA) and tissue-culture (TC) cells. Asterisk indicates one-sided u (number of runs) test. *See* pp. 287–289, *Introduction to Statistical Analysis*, 2d ed., by W. J. Dixon and F. J. Massey, 1957, McGraw-Hill Book Co.

to the usual lethal dose of 10^3 MA cells is shown by groups IB and IIB. While protection calculated on a basis of survival at 75 days after injection of the lethal dose was 50 percent in group IB and somewhat lower in group IIB, the median survival time in both groups was more than doubled over that of the controls.

DISCUSSION

Loss of virulence in the tissue-culture strain of Ehrlich ascites carcinoma apparently occurred rapidly, as shown by the low level of infectivity when doses of 10^3 and 10^4 cells were tested after 4 months' growth *in vitro* (12 subcultures). Concomitant with this loss of virulence is the ability of these tissue-cultured cells to induce some resistance or immune response. Doses of 10^3, when incapable of producing any visible ascites tumor in the mouse during a 6- to 7-week incubation period, will protect the mice who are inoculated subsequently with a lethal dose of 10^5 tissue-culture strain cells, and partially protect mice against an inoculation of 10^3 MA cells.

Our results, demonstrating the ability of TC cells after 10 months' *in vitro* cultivation to produce ascites tumors in Swiss mice, differ somewhat from the findings of Deschner and Allen (*13*), but do not necessarily contradict them. These authors found that after 55 days in tissue culture an inoculation of 4×10^3 cells failed to produce ascites tumors. Our experiments with concentrations of 10^5 to 10^7 as well as lower levels of TC cells showed that massive doses of the TC cells were needed to produce a high percent of tumors. In several series of experiments in

which a total of 75 mice was inoculated with either TC or TCS_1 cells at a level of 10^3, the transplantability averaged only 20 percent. When inoculation with TC cells is considered separately, only 4 positives were found in 30 animals, or approximately 13 percent.

Deschner and Allen also noted that their cells tended to grow on the roof or sides of the culture flask rather than in the medium or on the bottom surface of the glass. Their observations are in contrast to all other available reports (*3, 17*) and may be characteristic of the particular strain or clone which became established in their medium, since the Ehrlich ascites carcinoma is particularly variable in chromosome number, virulence, etc., as indicated by Hauschka and Levan (*19*).

The data compiled in table 1 are obviously limited in both the number and scope of experiments so that definitive conclusions are not warranted, but some interesting speculations are possible. In most of our early experiments it was necessary to produce enough cells to test a series of mice at levels of 10^6, 10^7 cells, etc. The only way this could be done was to inoculate 1 or 2 mice with a high dose of TC cells, and, when these mice had developed an ascites tumor, to take this first passage material (TCS_1) for inoculation into the test series (TCS_2). All experiments showed that 10^7 cells produce 100 percent tumors, and 10^6 cells average 80 percent tumors in both TCS_1 and TCS_2 mice. However, it is possible that during the first passage in mice the more virulent cells tended to multiply, while the less virulent ones were destroyed. These cells which had come from tissue culture and multiplied in the first mouse passage TCS_1 would then show greater virulence on subsequent transfer (TCS_2). Two series of studies seem to support this hypothesis. While all three experiments (A, B, C, table 1) were made into second passage mice, the cells grew for 12 days in the first passage animals in experiment C, as contrasted to 8 days in groups A and B. Results show that a somewhat greater degree of virulence, especially at a level of 10^3 cells, occurred in experiment C. Conversely, in experiment D in which TC cells were used, all levels tested show a marked decrease in virulence.

If one assumes that the loss of virulence of the cell line and increase in resistance or immune response of the host animal are concomitant, the data in text-figure 2 appear to support this hypothesis to some degree. While the number of mice developing tumors is practically identical whether inoculated with 10^3 TC or TCS_1 cells (4 vs. 5 out of 25), the resistant mice from group I receiving 10^3 TC cells exhibit a slightly greater immunity to both challenging doses of 10^3 MA cells or 10^5 TC cells than the mice in group II which received 10^3 TCS_1 cells.

While these preliminary studies on virulence have been made with only one line of tissue-cultured cells, the change or loss of pathogenicity is not unexpected. Hauschka and Levan (*19*), Klein and Klein (*20*), and Yoshida (*21*), among others, have shown that cloned tumor cells (in mouse-to-mouse passage but not in tissue culture) will vary greatly in their abilities to produce ascites tumors in various strains of mice. San-

ford *et al.* (*22*) have cloned several strains of mouse cells in tissue culture which present high and low capacities to produce tumors in the parental strain of mice. Guerin and Kitchen (*8*) have obtained a tissue-culture strain of the mouse ascites 6C3HED cells and find an altered virulence when these cells are reinoculated into Swiss (Connaught) or C3H mice.

Further studies with the multinucleate cloned strain of the Ehrlich ascites cells growing on ascitic fluid medium or other variants obtained *in vitro* are planned. Variations in the chromosome number or pattern undoubtedly affect the virulence of the cell and the response of the host. More detailed tests with definite or identifiable cell strains might help to elucidate the mechanism involved. Additional studies on the type of resistance or immune response provoked by the tissue-culture strains are planned, with both Swiss and inbred or genetically pure mouse strains.

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Plate 28

Figure 1.—Ehrlich ascites tumor cells growing in fetal calf-serum medium. Cells are predominately spindle-shaped. Polychrome stain. × 340

Figure 2.—Ehrlich ascites tumor cells growing in ascitic fluid medium. Cells tend to fuse, are ameboid and frequently multinucleate. Hematoxylin and eosin. × 340

Figure 3.—Chromosomes of cells grown on fetal calf-serum medium (68 chromosomes). Aceto-orcein stain. × 1,000

Figure 4.—Chromosomes of cells grown in ascitic fluid medium (approx. 130 chromosomes). Aceto-orcein stain. × 850

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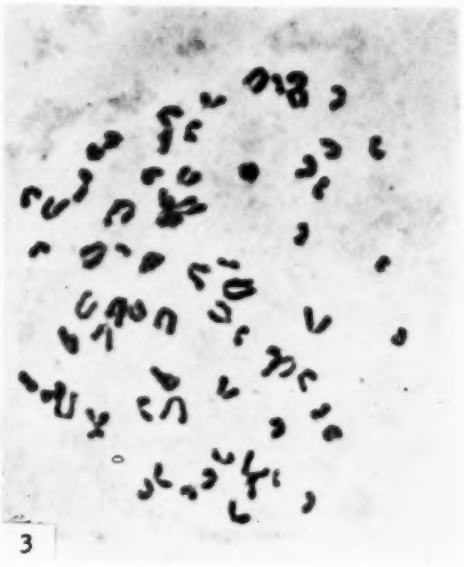

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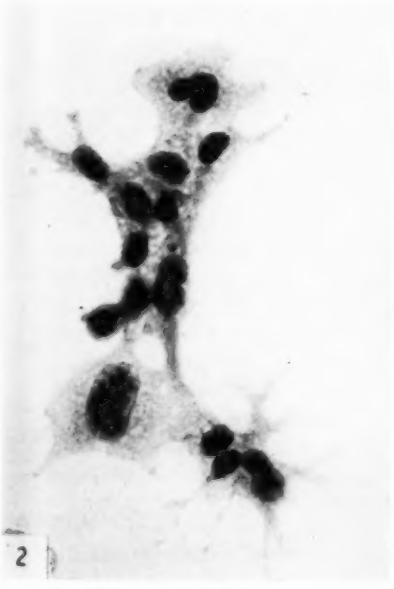

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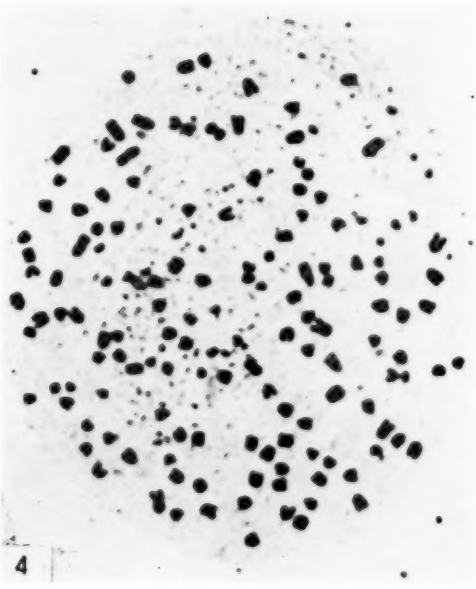

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Male Histocompatibility Antigens in Mouse Endocrine Tissues: Functional and Histologic Evidence [1, 2]

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SUMMARY

We have sought evidence of the male-linked histocompatibility antigens in parathyroid, thyroid, and adrenal cortical tissue by assessing the fate of endocrine fragments from male donors placed as isografts into adult female mice (after preimmunization with or concomitantly with male skin isografts known to contain the antigens). Serial serum calcium assays in previously parathyroidectomized mice receiving intramuscular parathyroid fragments invariably provided clear functional evidence of the destruction of the buried male parathyroid tissue. Histologically a specific mononuclear cell inflammatory reaction was noted in male-to-female isografts of all three endocrine tissues, in striking contrast to the appearance of corresponding female-to-female control isografts. Thus male histocompatibility antigens are present in these endocrine tissues, and no deficiency in the activity of these antigens as compared to those of skin was demonstrable. Some immunological characteristics of these male antigens which may limit the applicability of these results to other mouse transplantation antigens are discussed.—J. Nat. Cancer Inst. 26: 283–303, 1961.

WITHIN MANY inbred mouse strains, skin grafts from male to female are eventually rejected while male-to-male, female-to-male, and female-to-female skin grafts are permanently accepted. These observations originally made by Eichwald and Silmser (*1*) have been analyzed further (*2–8*), and there is wide agreement that the Y chromosome bears responsibility for determining the presence of antigenic material in the skin of male mice which is not present in that of females.

We have previously shown in rats that parathyroid cells, perhaps by virtue of their highly specialized function, may be relatively deficient in effective transplantation antigens as compared to the skin of the same animal (*9*). We found, for instance, that in several functionally and

[1] Received for publication July 28, 1960.

[2] Supported by research grant A-1578 from the National Institute of Arthritis and Metabolic Diseases and grant CRTY-5018 from the National Cancer Institute, National Institutes of Health, Public Health Service. This is publication No. 1029 of the Cancer Commission of Harvard University.

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histologically documented simultaneous transplants of skin and parathyroid tissue between Wistar albino rats, the endocrine tissue continued to function after complete rejection of both a first- and even a second-set skin graft from the original donor. We have here sought to narrow the genetic difference between donor and recipient animals and thereby to seek evidence for a specific antigenic deficiency in some highly specialized endocrine tissues. For this, we have used grafts between male and female members of the same inbred strains of mice as a readily available analogue of transplants between the "coisogenic" strains which differ at a single histocompatibility genetic locus (*10*).

In these experiments we have examined parathyroid, thyroid, and adrenal cortical tissue, carrying out intramuscular gland grafts simultaneously with orthotopic skin grafts from males to females within 2 inbred mouse strains. Similar grafts from females to males or between females served as controls. In some animals serial serum calcium microassays afforded a functional criterion of survival or rejection of parathyroid grafts, which was correlated with histologic findings. Elsewhere the fate of these buried grafts was determined by histologic examination at appropriate times. Invariably in male endocrine tissues we found evidence of the presence of antigens not found in otherwise isogenic female recipients.

METHODS

Adult mice of the A/Jax strain, 8 to 10 months of age, were used in parathyroid and thyroid grafting. C57BL/6 mice were used for the adrenal transplantation experiments.

Endocrine tissues were removed and cleared of extraneous connective tissue under the dissecting microscope. The minute bits of cleaned tissue were promptly implanted into intramuscular pockets prepared in the hamstring muscles of the recipient. This method previously resulted in prompt function of implanted adrenal (*11*) and parathyroid (*9*) tissue with no evidence of ischemic necrosis, if fragments of 1 to 2 mm in size were used. Except in those parathyroid grafts where function was to be assessed metabolically, a control parathyroid graft from another adult female isogenic with its recipient was implanted in an adjacent pocket in the muscle.

Skin grafts were prepared and applied (*12*) with either ear or abdominal donor skin. In each instance a skin graft from another female was placed adjacent to the male skin graft as a technical and histologic control.

Prospective recipients of parathyroid grafts to be followed functionally were first parathyroidectomized, most of their thyroid tissue being spared. They were then placed on a synthetic diet originally designed for rats (*13*) and modified by Munson[5] with the substitution of calcium-deficient salts. Protracted periods of hypocalcemia were tolerated with little obvious

[5] We gratefully acknowledge receipt of the low-calcium diet from Dr. Paul L. Munson, School of Dental Medicine, Harvard University, Boston, Mass.

neuromuscular irritability by these adult mice, similar to our previous experience with adult rats. Unlike the rat, however, the synthetic low-calcium diet induced a dermatitis with focal ulcerations in many of the mice, whether normo- or hypocalcemic. This eventually took a heavy toll of animals in all long-term experiments in which metabolic studies were done.

Calcium microassays were done on 0.1 ml serum samples by a modification of the EDTA-colorimetric method of Munson *et al.* (*14*). Blood samples of approximately 0.5 ml were obtained from the retro-orbital venous plexus by siliconed capillary pipettes. Mice were bled serially before and after grafting, but usually no more often than once a week, to avoid anemia.

The serum calcium levels of 20 percent of the 30 parathyroidectomized A/Jax mice maintained on the low-calcium diet continued in the normal range. We assumed this evidence of persistent parathyroid tissue to indicate an appreciable percentage of accessory parathyroids in this group of animals. All the animals with persistently normal calcium levels were excluded from further consideration.

For histologic observations paraffin sections were cut serially from excised blocks of hamstring muscle containing the endocrine grafts. They were first marked to keep track of the relative position of male to adjacent control female grafts, then scanned unstained for graft tissue before appropriate ones were stained with hematoxylin and eosin. Representative sections of the corresponding skin grafts were also prepared.

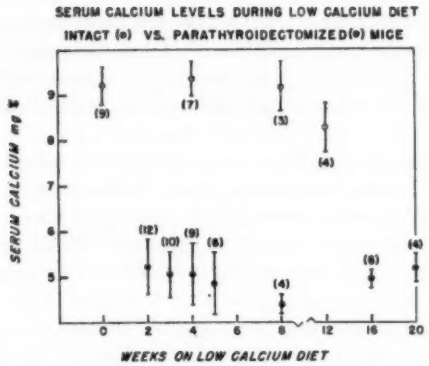
RESULTS

Parathyroid and Thyroid Experiments

Skin grafts from male to female of the A/Jax strain of this age were all rejected after intervals of 27 to 140 days, with a median survival of 45 days. These rejections were invariably of the "chronic" type marked by depilation, hyperkeratosis, punctuate ulceration, and contraction lasting from a few days to a few weeks. The controls behaved in every respect like autografts. The time of rejection was arbitrarily fixed at the day when graft contraction was first clearly noted. The typical histologic appearance is shown in figure 3.

Physiological limitations in the frequency of bleedings and the somewhat decreased accuracy of the calcium assay, with the necessarily small volumes obtainable, restrict the completeness and accuracy of this method of following the viability of parathyroid grafts in mice. Nevertheless the large differences between the serum calcium values of normal and parathyroidectomized mice, when both are maintained on the low-calcium diet (text-fig. 1), are so substantial that this method clearly affords good qualitative evidence of function.

Nine recipients survived the repeated bleedings, parathyroidectomy, and the low-calcium diet to the time when the fate of the functionally

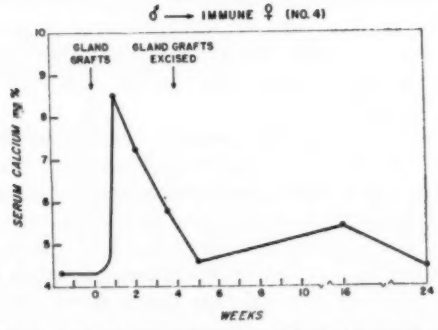

SERUM CALCIUM LEVELS DURING LOW CALCIUM DIET
INTACT (o) VS. PARATHYROIDECTOMIZED (•) MICE
SERUM CALCIUM mg %
WEEKS ON LOW CALCIUM DIET

TEXT-FIGURE 1.—Mean serum calcium values obtained by microassays of serums of normal and parathyroidectomized adult strain A mice maintained on a low-calcium diet. Each point is spanned by twice the standard deviation of the mean for the number of animals indicated in parentheses.

assessed grafts was apparent. In every case 4 parathyroid glands, twice the normal complement, were implanted after trimming most, but not all, of the surrounding thyroid.

Three females first received from male donors standard skin grafts, which survived for 42, 50, and 57 days. After this sensitization to male skin, all 3 were parathyroidectomized and, while on the low-calcium diet, received intramuscular parathyroid grafts from adult male A/Jax donors. Serum calcium assays indicated a period of transient graft function for about 2 weeks in all animals, suggestive of a survival time similar to that encountered with second-set grafts of male skin. Text-figure 2 shows the changes in calcium levels of 1 of these. (The histologic picture of grafted parathyroid removed from this animal appears in fig. 5.)

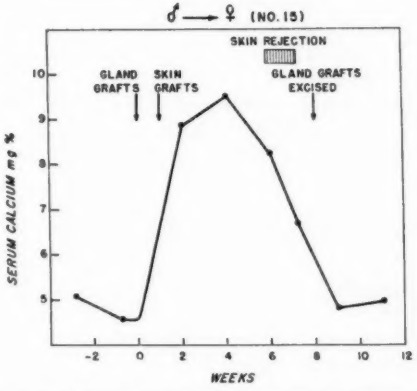
Three females received male skin and parathyroids at the same time. Function of the parathyroid grafts apparently ceased as the skin was



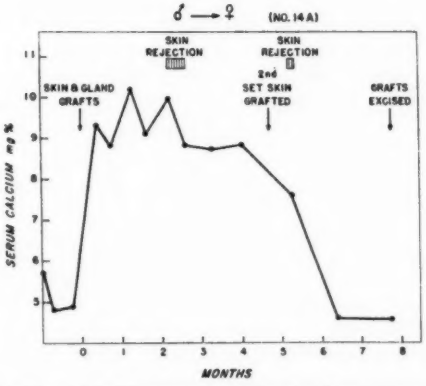
TEXT-FIGURE 2.—Serum calcium changes indicating accelerated destruction of a male parathyroid isograft in a female which had previously rejected male skin after 50 days. *See* figure 5 for the histology of the excised graft.

rejected in 2 of them 40 and 45 days later. One of these is illustrated in text-figure 3 and figure 2. In the third animal the grafted parathyroids showed persistent evidence of function after the slow skin graft rejection at 70 days was complete. Full rejection of the parathyroid grafts occurred, however, when a "second-set" male skin graft was applied and rejected in the expected 16 days. The serum calcium values of this latter animal appear in text-figure 4, its histology in figures 7 and 8.

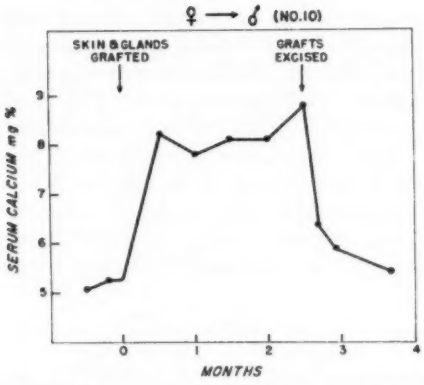
Three parathyroidectomized male controls were grafted with female skin and parathyroids. Serum calcium levels in these animals remained in the normal range until the intramuscular glands were excised months



TEXT-FIGURE 3.—Serum calcium changes after concomitant grafts of strain A male skin and parathyroid glands in a previously parathyroidectomized strain A female. The "chronic" skin rejection coincides with the cessation of parathyroid function. *See* figures 2 and 3 for the histology of the rejected skin and glands.



TEXT-FIGURE 4.—Serum calcium changes after simultaneous grafts of male skin, thyroid, and parathyroid glands in another previously parathyroidectomized isologous strain A female. The immunity elicited by a "second-set" male isograft was needed to bring about cessation of parathyroid graft function. *See* figures 7 and 8 for the histology of the parathyroid and thyroid grafts excised.



TEXT-FIGURE 5.—Serum calcium determinations in a parathyroidectomized strain A male receiving grafts of isologous female skin and parathyroid glands. Late excision of the grafts was followed by fall of serum calcium to baseline levels. *See* figure 1 for the histology of the excised gland grafts.

later, as can be seen in text-figure 5, showing the serum calcium values of 1 of them.

Additional information was obtained from 6 other recipients of thyroid-parathyroid implants, evaluated histologically: 1) Three females received male skin, thyroid, and parathyroid tissue, with adjacent grafts of female tissue as histologic controls. The male skin was in an advanced stage of rejection at 48, 48, and 58 days, respectively, at which times all grafts were excised for histology. 2) Three females which had previously rejected both skin and parathyroid from male donors received simultaneous "second-set" male skin, parathyroid, and thyroid grafts, along with control grafts from female donors. When the male skin grafts were being rejected at 15, 15, and 16 days, respectively, all grafts were again excised for histology.

Histologic examination of the implanted female parathyroid and thyroid tissue in male or female recipients consistently revealed normal gland architecture, with no cellular infiltration and no inflammation at the host-graft boundary, as is shown in figure 1.

First-set male grafts invariably showed some inflammatory cellular infiltration which plainly differentiated the male from the female grafts. Figure 4 illustrates the contrast in the thyroid tissue. The typical first-set reaction to parathyroid tissue associated with cessation of function is shown in figure 2, where disruption of normal parathyroid architecture can be seen in comparison to a control graft in figure 1.

Examination of second-set grafts in these experiments revealed a more rapid and intense reaction as seen in figure 6, which shows a portion of a second-set male to female thyroid graft at 15 days after implantation.

The reactions against male thyroid and parathyroid grafts were not always of consistent severity. Thus in the recipient whose calcium values are plotted in text-figure 4, the primary reaction obviously allowed

its endocrine function to continue. Eventually the typically vigorous rejection of a superimposed second-set skin graft at 16 days coincided with the cessation of parathyroid function. Some sections of male thyroid and parathyroid grafts, excised nearly 8 months after implantation, showed that by that time the parathyroid tissue had been completely replaced by inflammatory cells, as shown in figure 7, while male thyroid tissue implanted in the same animal evoked only the milder reaction seen in figure 8.

Adrenal Cortex Experiments

C57BL/6 females were grafted with both male and female adrenal cortical shavings of the same inbred strain. These were implanted either after immunization by or concomitant with male C57BL/6 skin grafts.

The adrenal shavings were recovered for histologic examination from 7 recipients. An example of a female isograft control is seen in figure 9. Plasma cells were not seen about the control grafts. The male adrenal grafts encountered a distinct reaction involving many lymphocytes and plasma cells, suggestive of its immunological nature. An illustrative example is shown in figure 10, where only occasional distorted adrenal cells and the outline of the adrenal capsule can be discerned through the population of infiltrating cells.

DISCUSSION

Previous studies have suggested that male-determined histocompatibility antigens are present in normal tissues other than skin. Thus Eichwald, Silmser, and Weissman (*7*) reported that most subcutaneous implants of normal male lung and salivary gland within the C57BL strain showed histologic evidence of rejection in isologous females. Again, in the course of experiments evaluating a possible hormonal factor in the "Eichwald-Silmser phenomenon," these authors found that isologous testicular grafts to C57BL females, though not examined histologically, significantly decreased the survival of subsequent male skin grafts, which suggests the presence of male antigens in testicular tissue. Lymph-node and spleen suspensions (*15*) as well as thymus cells (*16*) have been shown to contain the Y-determined antigens.

The present experiments failed to demonstrate a specific deficiency of these male antigens in the mouse parathyroid, thyroid, and adrenal tissues studied. These results are consistent with the fairly extensive evidence favoring the similarity of antigenic complement of various normal tissues reviewed by Medawar and Russell (*11*). The recent findings of Hicken and Krohn (*17*), that approximately the same number of genetic loci control the histocompatibility antigens responsible for rejection of both skin and ovarian grafts in some mice, also support this conclusion.

It may well be that the male antigen system selected in these experiments is not a close analogue of the other well-studied transplantation

antigens in the mouse, such as those determined by the various *H* loci described by Snell. Ingenious experiments with hybrid strains have shown the Y-determined antigens to be present and identical in all strains tested (*7*, *8*). This situation differs from the *H* loci-determined antigens which may be present or absent according to their behavior in Mendelian segregation, where they act as dominant traits (*10*), and indicates a less complex generic locus than, for example, the *H–2*. C57BL and A/Jax adult females have been reported to remain tolerant or unresponsive to adult male skin grafts after a period of parabiosis with males, and isologous male spleen cells can induce tolerance of male skin grafts if first injected as late as 3 months after birth (*18*). Billingham has recently found (*19*) that the neonatal injection of male spleen cells from homologous mouse strains into C57BL females regularly produces tolerance of male C57BL skin grafts, which suggests that the male antigen can induce a persistent tolerance in the absence of self-perpetuating, antigen-producing homologous cells. Also recently Hauschka *et al.* (*20*) failed to demonstrate hemagglutinins, though weak leuko-agglutinins and leukocytotoxins were present in their hyperimmune isologous anti-male serums. Most of these observations have no parallel in previous work with homologous antigen systems.

Thus the present evidence lends no additional support to our previous contention, based on experiments in the rat (*9*), that parathyroid tissue can differ from skin in its apparent complement of transplantation antigens, a concept which might also explain the findings of Billingham and Parkes (*21*), who studied ovarian grafts in the same species. A full explanation of the discrepancy in behavior between different tissues of the same animal is not yet available. Further studies with coisogenic donor-recipient pairs may be helpful in clarifying this issue.

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PLATES

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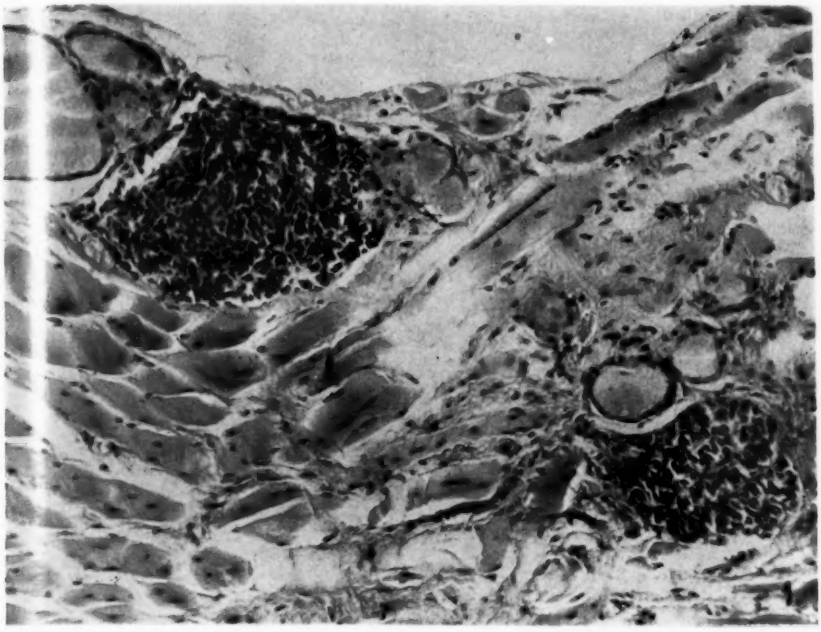
PLATE 29

FIGURE 1.—Control female isografts of parathyroid tissues with attached tabs of thyroid, fully functional when excised from A/Jax male host as shown in text-figure 5 (No. 10). × 125

FIGURE 2.—Nonfunctioning male parathyroid removed at 57 days from isologous female recipient after cessation of function illustrated in text-figure 3, coincident with the primary reaction to male skin shown in figure 3 (No. 15). × 125



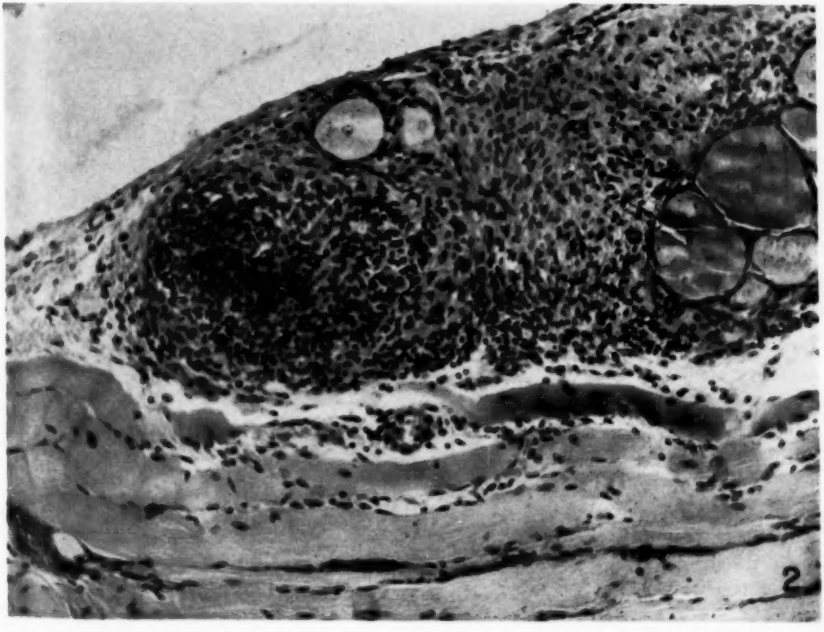


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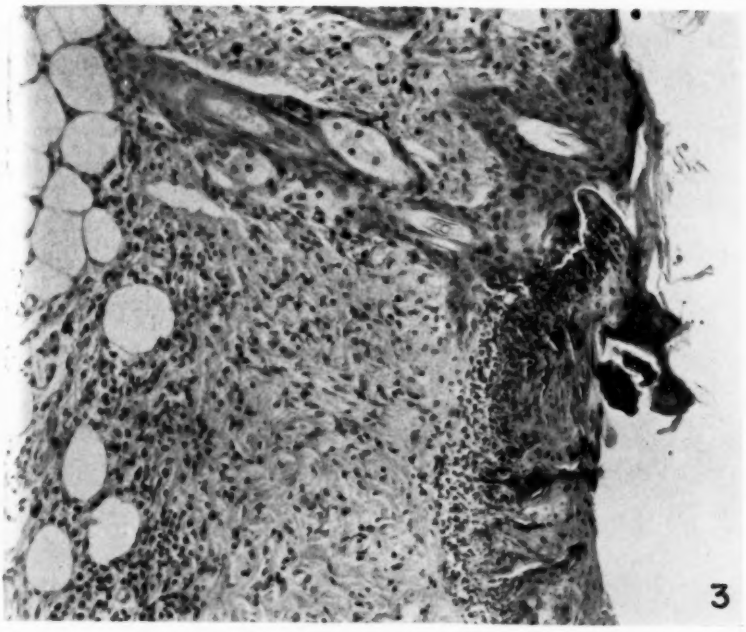
PLATE 30

FIGURE 3.—Primary reaction to male skin isograft biopsied at 45 days in the female recipient illustrated by text-figure 3 and figure 2. Control female isograft is seen *above* and adjacent to rejected male isograft (No. 15). × 125

FIGURE 4.—Primary reaction to male thyroid isograft on *right* with adjacent female control on *left*, excised from female recipient at 58 days when concomitant male skin isograft was rejected (No. 1T). × 200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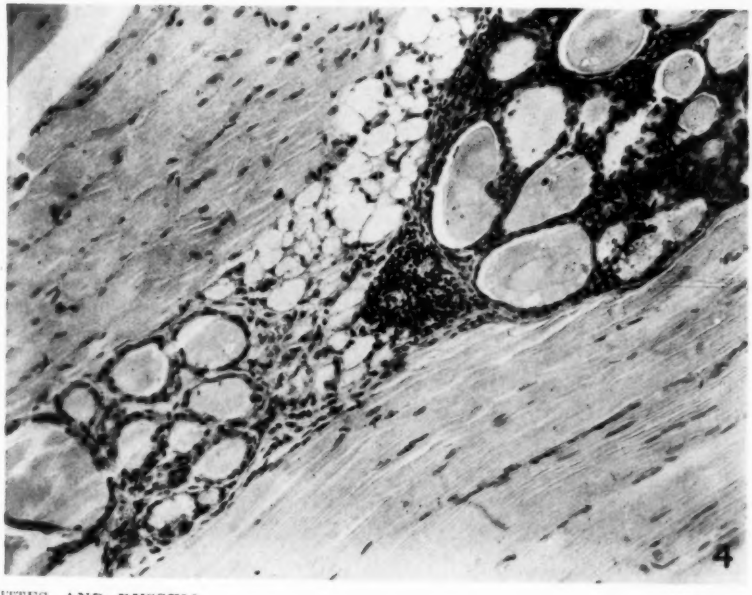
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PLATE 31

FIGURE 5.—Secondary reaction to male parathyroid isograft excised at 30 days from female recipient when cessation of function was evident, as illustrated in text-figure 2. Heavy mononuclear infiltration and loss of parathyroid architecture (No. 4). × 125

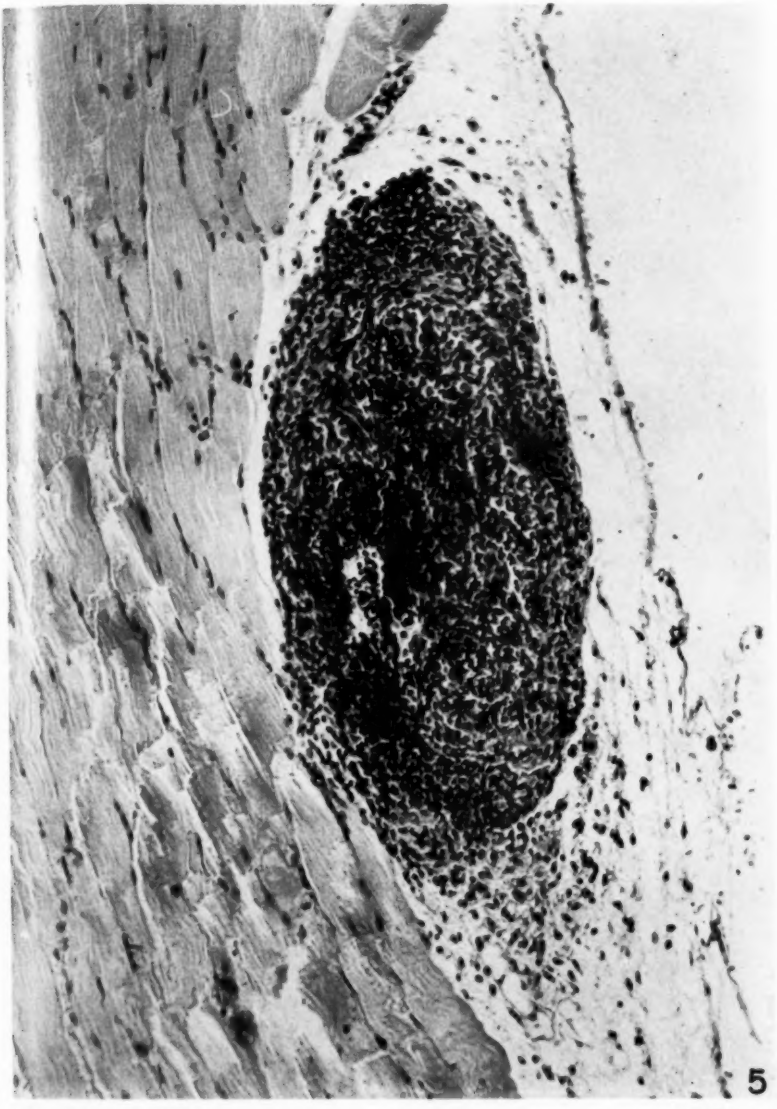
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PLATE 32

FIGURE 6.—Violent secondary reaction to male thyroid isograft excised at 15 days when concomitant second-set male skin isograft was rejected (No. 13T). × 200

FIGURE 7.—Reaction against first-set parathyroid excised as indicated in text-figure 4 after cessation of its function coincident with rejection of second-set male skin. No parathyroid cells are identifiable (No. 14A). × 125

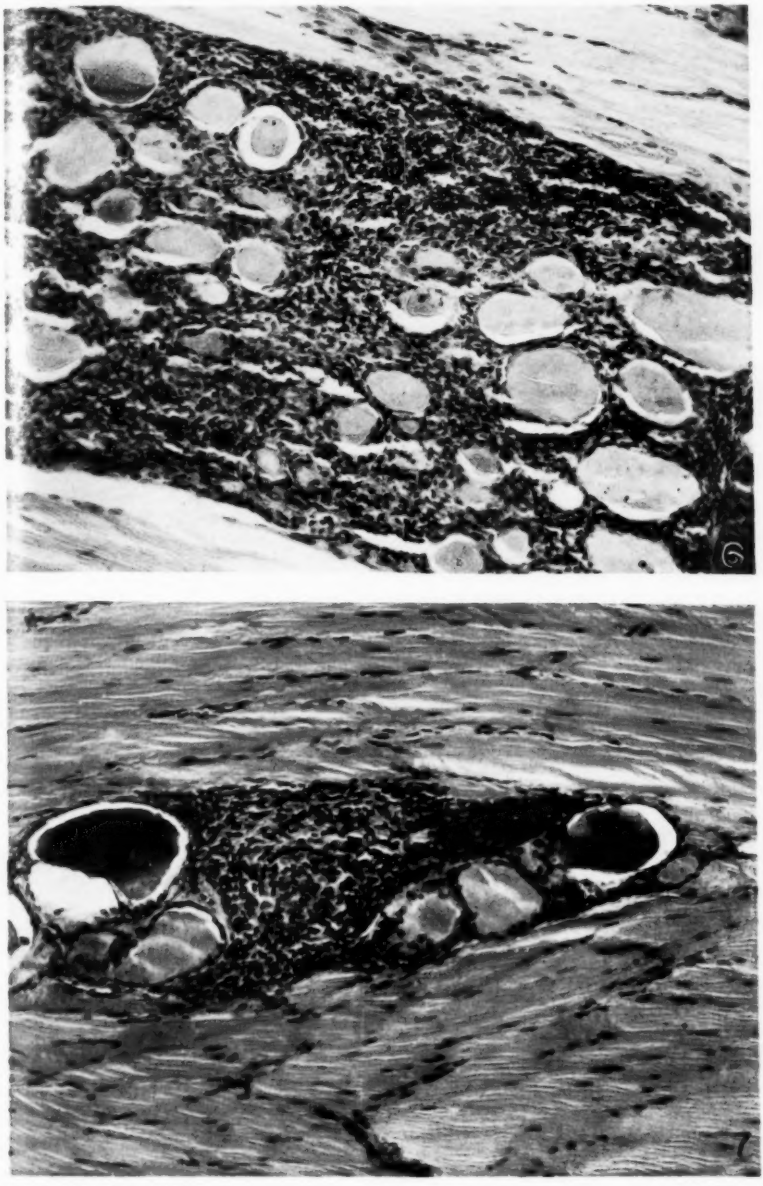
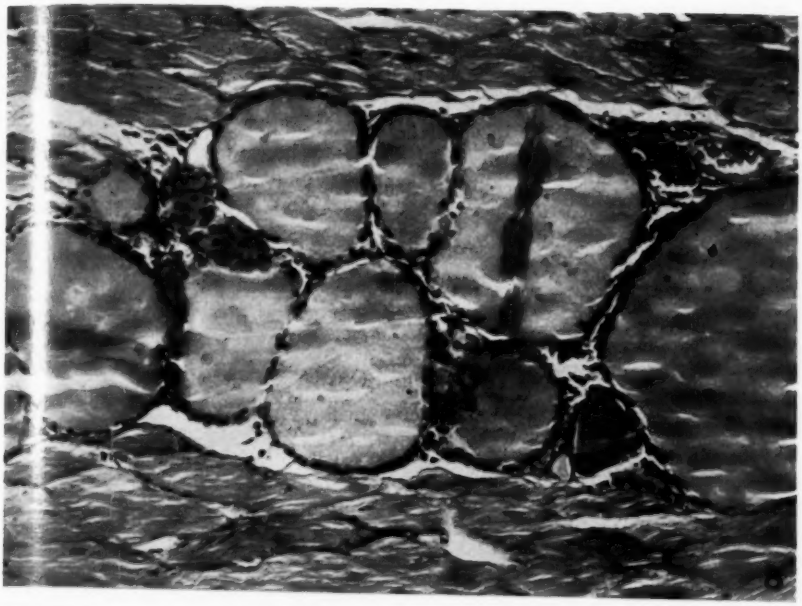


PLATE 33

FIGURE 8.—Mild reaction against the thyroid component of male isografts excised nearly 8 months after implantation in female mouse, represented also in text-figure 4 and figure 7. Flattened but persistent epithelium and good colloid are noted, with some interstitial infiltration (No. 14A). × 125

FIGURE 9.—Control female to female adrenal isograft excised at 34 days. Cellular morphology of fragment nearly intact (No. 7–1). × 125



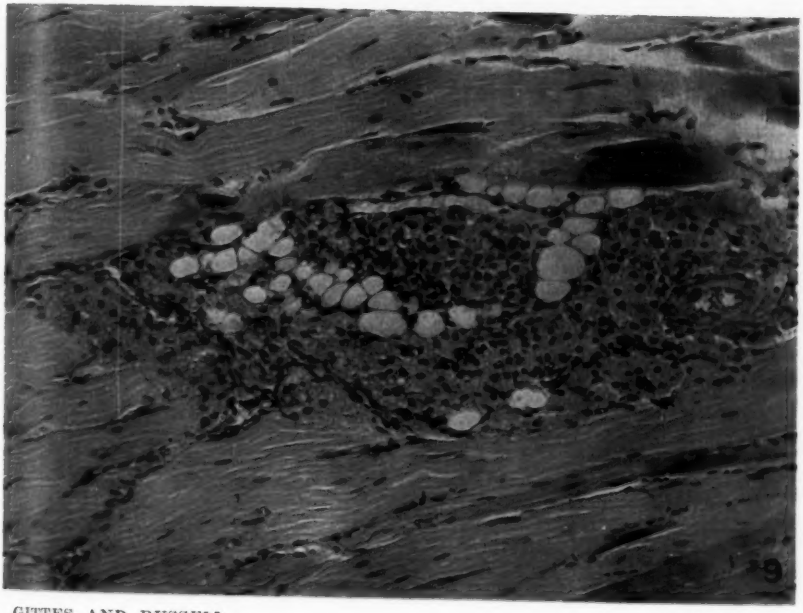
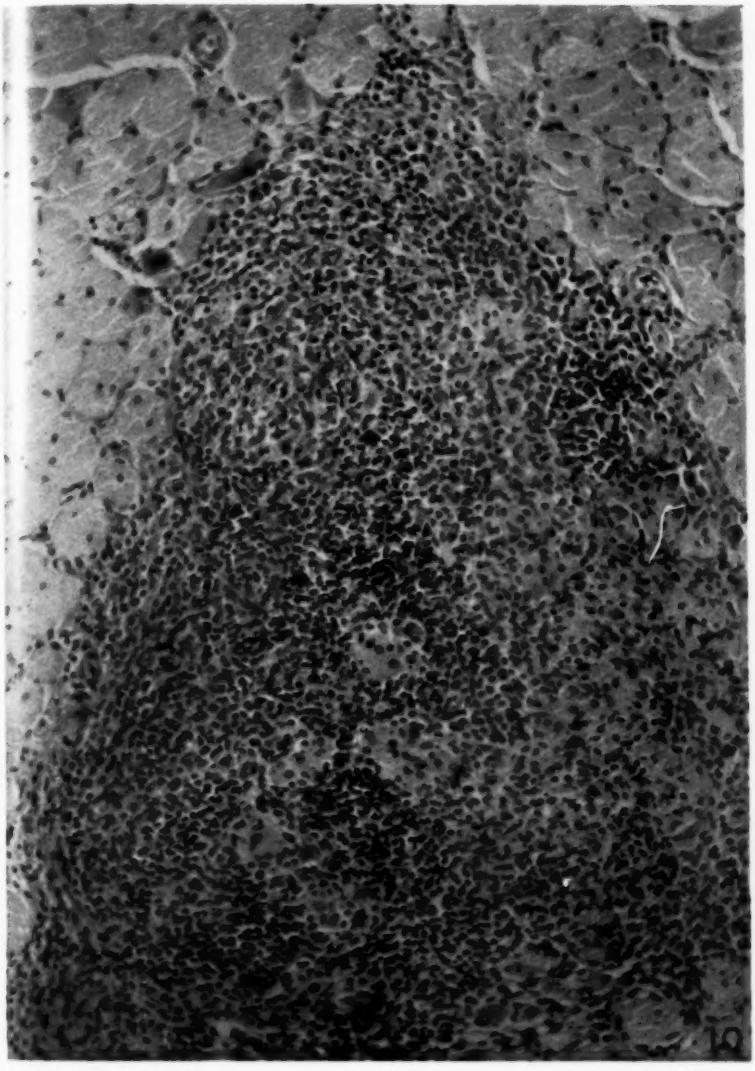


PLATE 34

FIGURE 10.—Adrenal male to female isograft excised at 34 days from recipient rejecting concomitant male skin isograft. Strong reaction seen with only occasional distorted adrenal cells glimpsed among infiltrating cells (No. 7–1). × 125



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Pancreas Passage of Rous Sarcoma [1]

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SUMMARY

Serial intracoelomic passage of a strain of Rous sarcoma virus, obtained from Dr. W. Ray Bryan, National Cancer Institute, Bethesda, Maryland, showed the pancreas to be a novel, reliable source of virus for experimental purposes. Intracoelomic injection of pancreas-passed Rous sarcoma virus resulted in a relatively high and reproducible mortality in 8-day-old cockerels within a test period of 22 days. A significant regression in ED50 ($P = 0.01$) was observed over a series of 30 consecutive passages. The effect of the infection appeared to be a fulminating, ravaging disease, capable of invading and damaging almost any vital organ, with death as the end result.—J. Nat. Cancer Inst. 26: 305–313, 1961.

SINCE ITS isolation by Rous (*1*), most of the work with the Rous sarcoma virus has been performed with tissues or filtrates obtained from localized tumors. Vázquez-López (*2*) showed the brain of chicks to be a suitable tissue for virus propagation, an observation which was later confirmed and expanded by Groupé *et al.* (*3*). The general observation that the virus can exist in most tissues of the infected bird has not been extensively explored because the concentration of virus normally found in these tissues was of a very low order (*4*). The work in this report was prompted by a chance observation of severe sarcomatous involvement of the pancreas in a bird that died after an intracoelomically induced infection with Rous sarcoma virus (RSV). The possible utility of infected pancreas tissue for the induction of a lethal infection was examined. The results obtained with serial passage of infective pancreas tissue are herewith presented.

MATERIALS AND METHODS

Animals.—The 8-day-old Hallcross sex-linked cockerel was used as the standard experimental animal in all tests. The chicks were received when 1 day old and were housed in heated battery brooders until time of test.

[1] Received for publication August 1, 1960.
[2] We wish to express our thanks to Dr. William F. Kwolek for his statistical analysis of the data.

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While on test, the chicks were kept in groups of 10 in wire cages at a controlled room temperature of 80 to 90° F and under artificial light on a 12-hour-on, 12-hour-off cycle. The groups were composed on a weight basis so that the average weight per bird from group to group varied no more than ± 2 g. The weight range for individual birds was 55 to 65 g. The birds were kept on standard broiler mash throughout the test and had access to both feed and water at all times.

Source of virus.—RSV I, lot CT 694, obtained from Dr. W. Ray Bryan, National Cancer Institute, Bethesda, Maryland, was used to initiate this series of experiments. This material was a partially purified suspension of Rous sarcoma virus prepared from a pool of wing-web tumors (*5*).

Preparation of inoculum.—The pancreas from chicks infected intracoelomically with a 10^{-3} (100–1000 ED50) dilution of virus suspension was used as the source of virus for the preparation of all inoculums.

The harvest time of infective pancreas tissue varied from 11 to 14 days after infection during the first 9 passages, and was 13 days for all subsequent passages. All birds used for source of inoculum were killed by dislocation of the cervical cord.

Pancreas tissue to be used for the purpose of passage was taken only from birds that showed severe and spreading tumor growth and whose pancreas showed gross enlargement and distortion, associated with mucoid degeneration and hemorrhage (fig. 1). The inoculum was prepared as follows: Pooled infected pancreas tissue was weighed and then homogenized for 2 minutes in a Waring blendor in sufficient brain-heart infusion broth to make a 10 percent suspension on the basis of weight. After standing for 2 hours in a refrigerator at 3 to 5° C, the suspension was centrifuged for 15 minutes at 1000 × *g*. The supernatant was saved for purposes of infection and the sediment was discarded. The undiluted supernatant was referred to as the 10^{-1} dilution of pancreas tissue. Further dilutions were made in brain-heart infusion broth. All suspensions were tested for sterility by inoculation of 0.2 ml of the 10^{-1} dilution into 10 ml of thioglycolate broth and incubation at 37° C for 48 hours. Inoculums were stored in sealed vials in a dry-ice chest at −70° C after having been quick-frozen in an acetone dry-ice bath.

Route of infection.—All inoculations were given intracoelomically. The standard inoculum was 0.2 ml of a given dilution per chick. The inoculum was placed within the vicinity of the duodenal loop with a ½ inch, 27-gauge needle. This small-gauge needle was found to reduce subcutaneous leakage of the inoculum and concomitant subcutaneous tumor growth to a minimum.

Criteria for specificity of deaths.—Deaths which were not associated with any evidence of neoplastic involvement were considered as nonspecific. These deaths usually occurred during the 1st week of the test period and averaged 1.5 to 2.5 percent for both the infected and the sham-inoculated control birds. It is assumed that these were probably caused by trauma or stress of handling. These nonspecific deaths were included in the experimental mortality data.

Specific deaths were always associated with neoplastic involvement, hemorrhage, or both. Neoplastic involvement and hemorrhage were assessed by postmortem examination; however, at times neoplastic involvement was determined by simple palpation.

The median survival time (ET50) for the total bird population of these experiments was calculated for each infecting dilution by the method of Litchfield (*6*).

EXPERIMENTAL RESULTS

Infectivity of Initiating Virus

Initial experiments showed that wing-web tumors could be obtained in Hallcross cockerels with the injection of 0.2 ml of a 10^{-5} dilution (highest dilution tested) of Bryan's RSV, lot CT 694.

Twenty chicks were inoculated intracoelomically with 0.2 ml of a 10^{-2} dilution of the virus and observed daily. Two of these chicks developed palpable tumors on the 12th day after infection and were killed; 6 died with a generalized tumor infection in 12 to 19 days, and 12 survived for 28 days and were killed. Autopsy of the 12 birds which survived for 28 days showed that 3 had no macroscopic lesions, 8 had well-defined tumors in the liver, and 1 exhibited severe and widely disseminated tumor growth. The 2 chicks with palpable tumors killed on the 12th day were observed on autopsy to have tumor growth in the pancreas. The pancreas tissue from these chicks was used as the starting material for 30 consecutive passages, in each of which the infected pancreas was used as the source of virus.

General Observations of Birds Infected Intracoelomically With Pancreas-Passed RSV

For 1 week after infection the birds showed no overt signs of being ill. Average weight gains and feed consumption were similar for infected birds and noninfected, sham-inoculated control birds. During the 2d week, the infected birds showed a decrease in feed consumption which varied with the infecting dose, the largest dose causing the greater decrease. Simultaneously with reduced feed intake, drowsiness and listlessness became progressively evident, and tumor growth could be detected by palpation. The disease progressed rapidly, terminating in death, with, however, occasional prolonged lingering. Autopsies of dead birds, except those dying with hemorrhage, showed a somewhat varied but always severe tumor growth.

Tumor lesions were found to be well disseminated. Occasional tumors were found in the heart, testes, kidneys, and spleen. Lung tumors varied from isolated foci to tumors seeming to obliterate practically all normal tissue. Tumor involvement of the liver also varied from small, discrete lesions to large tumors, which at times reduced the normal-appearing tissue to a residual 25 percent or less.

The predominant autopsy finding was the severe involvement of the pancreas. The organ appeared enlarged and distorted with little semblance of the normal in either size or shape. A mucoid breakdown of the tissue streaked with blood was frequently seen. Heavy tumor growth in the mesentery was almost always associated with lesions in the pancreas. This mesenteric involvement frequently had advanced toward fusing the intestines into a solid, tumorous mass bathed in a yellow-brown or greenish, thick, glutinous fluid.

The incidence of hemorrhagic deaths was low and became evident only after the virus had been passed intracoelomically as pancreas homogenate for 15 passages. In these cases the pectoral muscle appeared to be dotted with bundles of fine, threadlike, red streaks parallel to the muscle fibers. Petechial hemorrhages were found in the mesentery, pancreas, intestines, liver, and spleen. Fatal hemorrhage occurred as rupture of the spleen, liver, or major blood vessel. Macroscopic evidence of neoplasia was frequently lacking in birds dying with hemorrhage.

Lethal Effect of Pancreas-Passed RSV in Intracoelomically Infected Chicks

Twenty-four of 30 consecutive passages were tested in 100-fold dilutions for their ability to kill 8-day-old cockerels within a test period of 22 days. Table 1 gives the combined daily deaths and the cumulative percent mortality of all experiments. The average percent mortality for all birds used in these tests was 91.1 for the 10^{-1}, 80.5 for the 10^{-3}, 62.5 for the 10^{-5}, and 21.7 for the 10^{-7} infecting dilution. There was a significant increase in survival time with each decrease in the infecting dose ($P = 0.05$). The progression was from 13 days for the 10^{-1}, to 15.2 for the 10^{-3}, to 18 days for the 10^{-5} infecting dilution.

Table 2 shows the mortality data as observed in the individual passages. These data were examined statistically to determine whether or not an increase in virus potency had occurred with continuous serial passage. An ED50 was calculated for each passage by use of the percent mortality for each dilution and the method of probits (*7*) as programmed for an LGP 30 electronic computer. The utility of the probit method of graphic analysis has recently been illustrated in detail for tumor latent periods (*8–10*) and time-to-death response (*11*, *12*). The weighted (with the reciprocal of the variance of the ED50) regression of log ED50 with passage number was significant at the 1 percent level.

The following regression equation was obtained:

$$\log \text{ED50} = -2.828 - 0.132x$$

where x is the passage number. When antilogs are taken

$$\text{ED50} = 1.49\ (10^{-3})\ (0.739)^{x}$$

The equation indicates that with each successive passage, up to 30, an average of 0.739 times (95% confidence limits 0.641 to 0.851) as much

TABLE 1.—Daily mortality and accumulated percent mortality of chicks infected with pancreas-passed RSV

Days after infection	Infecting dilution							
	10^{-1}		10^{-3}		10^{-5}		10^{-7}	
	Number dead	Accumulated % dead	Number dead	Accumulated % dead	Number dead	Accumulated % dead	Number dead	Accumulated % dead
1–7	17	2. 3	11	1. 4	10	1. 6	4	1. 1
8	25	5. 7	2	1. 7	1	1. 7	1	1. 4
9	33	10. 2	6	2. 8	1	1. 9	0	1. 4
10	51	17. 2	18	4. 9	2	2. 2	0	1. 4
11	88	29. 2	49	11. 4	3	2. 7	0	1. 4
12	83	40. 5	70	20. 5	7	3. 8	0	1. 4
13	78	51. 2	77	30. 7	15	6. 2	1	1. 7
14	78	61. 8	76	40. 7	47	13. 8	1	2. 0
15	66	70. 8	65	49. 2	55	22. 4	5	3. 4
16	42	76. 5	54	56. 3	52	30. 6	6	5. 1
17	33	81. 0	64	64. 8	74	42. 4	8	7. 4
18	28	84. 7	59	72. 5	43	49. 2	12	10. 9
19	20	87. 6	33	76. 8	40	55. 6	15	15. 1
20	11	89. 1	15	78. 8	26	59. 7	8	17. 4
21	11	90. 6	7	79. 7	12	61. 6	7	19. 3
22	4	91. 1	6	80. 5	7	62. 5	8	21. 7
D/T	668/733	91. 1	612/760	80. 5	395/630	62. 5	76/350	21. 7
ET50 (days)	13 (12.6–13.4)*		15.2 (14.7–15.7)		18 (17.5–18.5)		> 22	

*Figures in parentheses show upper and lower confidence limits at $P = 0.05$.

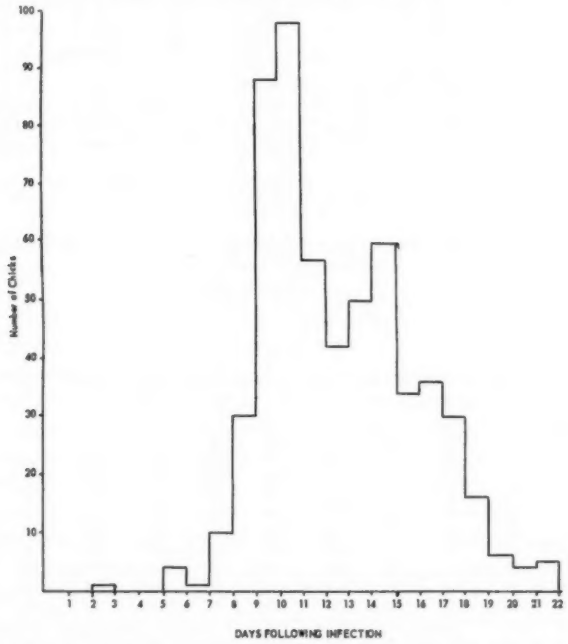
TABLE 2.—Lethal effect of pancreas-passed RSV in 8-day-old Hallcross cockerels

Passage number	Infecting dilution											
	10^{-1}			10^{-3}			10^{-5}			10^{-7}		
	Dead	Total	% Dead	Dead	Total	% Dead	Dead	Total	% Dead	Dead	Total	% Dead
2	6	9	67	5	10	50	4	10	40	—	—	—
3	12	14	86	10	10	100	—	—	—	—	—	—
4	8	10	80	8	10	80	1	10	10	—	—	—
5	10	10	100	8	10	80	8	10	80	—	—	—
6	44	50	88	39	50	78	—	—	—	—	—	—
7	31	50	62	13	50	26	—	—	—	—	—	—
8	26	30	87	25	30	83	13	30	43	4	30	13
9	48	50	96	43	50	86	20	50	40	3	50	6
10	27	30	90	24	30	80	21	30	70	2	20	10
13	28	30	93	21	30	70	21	30	70	2	20	10
14	25	30	83	23	30	77	17	30	57	6	20	30
15	29	30	97	24	30	80	14	30	47	1	20	10
17	—	—	—	28	30	93	16	30	53	6	20	30
18	30	30	100	28	30	93	18	30	60	4	20	20
19	28	30	93	27	30	90	23	30	77	2	20	10
20	28	30	93	22	30	73	15	30	50	3	20	15
22	30	30	100	23	30	77	18	30	60	2	20	10
24	29	30	97	24	30	80	21	30	70	8	20	40
25	28	30	93	24	30	80	22	30	73	10	20	50
26	29	30	97	29	30	97	28	30	93	10	20	50
27	29	30	97	26	30	87	23	30	77	5	20	25
28	46	50	92	44	50	88	32	50	64	—	—	—
29	48	50	96	48	50	96	33	50	66	—	—	—
30	49	50	98	46	50	92	27	30	90	6	10	60

tissue suspension was necessary to produce an effect equivalent to that of the previous passage. A similar statistically significant regression in survival time could not be shown.

Lethal Effect of 10^{-2} Dilution of Rous Sarcoma Pancreas Homogenate

The 10^{-2} dilution of infective pancreas homogenate was examined in 600 chicks for its ability to kill. Four late passages (26 and 28–30) were used for infecting material. The mortality and mean survival data for the 10^{-2} dilution are practically indistinguishable from those observed for the 10^{-1} infecting dilution in the more advanced passage numbers. The percent mortality of the combined experiments was 95.3 and the ET50 for the entire group was 12.5 days, with limits of 12 to 13 days ($P = 0.05$). The daily mortality is presented graphically in text-figure 1.



TEXT-FIGURE 1.—Frequency distribution of deaths in 600 birds infected with a 10^{-2} dilution of pancreas homogenate. Dead/total = 572/600 (95.3%), ET50 (days) = 12.5 (12–13).

DISCUSSION

The experiments described in this communication represent the preparatory efforts in establishing a lethal tumor-virus test system suitable for chemotherapeutic testing. The criteria set up for a suitable test model

were a routinely attainable mortality of better than 90 percent within a test period of 3 weeks, and death associated with rapidly metastasizing tumor growth. The long-established virus etiology of Rous sarcoma (*1*)—its tendency to metastasize and its utility in chemotherapeutic testing in intramuscularly (*12–14*) and subcutaneously (*15–18*) induced infections—motivated the choice of this agent for this study. It was felt that a lethal infection with this virus accompanied by metastasis might lead to information not otherwise obtainable and would supply data as meaningful as those obtained in other test systems. The effect of an active drug in such a test system could be the prevention or the delay of death as caused by the inhibition of virus multiplication and/or metastasis.

The data and observations presented indicate that through serial passage the pancreas of intracoelomically infected birds has been developed into a reliable source of virus. Special reference is made to the data obtained with the 10^{-2} infecting dilution in 600 chicks showing a 95 percent mortality within 3 weeks. The significant regression in ED50 ($P = 0.01$) with successive passages might reflect qualitative or quantitative changes in the inoculums; however, the available data do not permit a valid classification of these changes. The only conclusion which can be drawn is that it is unlikely that continued pancreas passage would result in a loss of the lethal effect of this virus, which thus assures a certain element of stability and reproducibility to this test system.

Chemotherapeutic studies with known antitumor agents are now in progress and will be the subject of a future publication.

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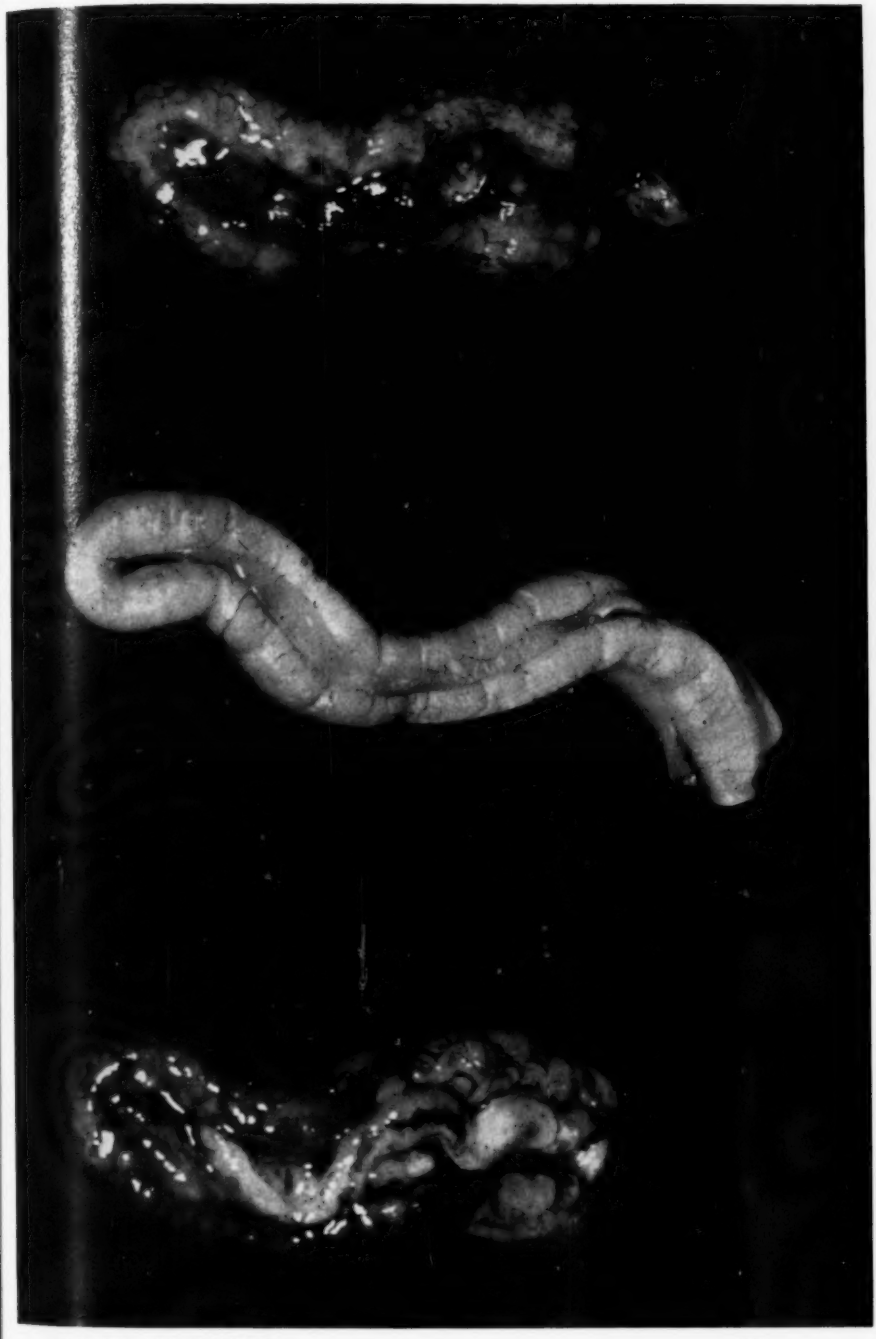
PLATE 35

FIGURE 1.—Chicken pancreas as situated within duodenal loop. *Center:* Tissue as seen in noninfected bird. *Upper* and *lower*: Tissues as seen in infected birds, judged suitable for purpose of virus passage.



An Effect of *N*-2-Fluorenylacetamide on the Metabolism of Tryptophan in Rats [1, 2]

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SUMMARY

Rats ingesting *N*-2-fluorenylacetamide (2-FAA) in a diet adequate for their maintenance and growth excrete in the urine increased amounts of free and/or conjugated xanthurenic acid, kynurenic acid, and hydroxylated kynurenine after a challenging dose of L-tryptophan. The rats excrete considerably less, or no detectable amounts, of these metabolites of tryptophan, if they are given small supplements of pyridoxine. This effect of 2-FAA is dependent on the concentration of the carcinogen in the diet, the amount of dietary pyridoxine, and the amount of tryptophan used for challenge. In repeated tests from the 10th to the 90th day of feeding there appeared to be no noticeable increase in the effect. During the early precancerous period when these tests were made, no macroscopic changes were usually seen in the liver, and no clinical evidence of B_6 deficiency was observed. Ingestion of carcinogenic amounts of *N*-2-fluorenyldiacetamide, a potent liver carcinogen, also interfered with the normal metabolism of a load dose of tryptophan, whereas the nonhepatocarcinogen, *N*-(7-hydroxy-2-fluorenyl)-acetamide, even at twice the molar equivalent level, had no apparent effect. There is indirect evidence that the accumulation of increased amounts of xanthurenic acid, kynurenic acid, and kynurenine derivatives may be due to an inhibition of kynureninase activity resulting from combination of the coenzyme pyridoxal phosphate with fluorenamine or some other amino metabolite of 2-FAA. Excess kynurenine coming from a tryptophan challenge may also inhibit kynureninase activity. The findings suggest that more than the ordinary requirement of vitamin B_6 may be needed by rats ingesting amino carcinogens.—J. Nat. Cancer Inst. 26: 315–329, 1961.

AN EFFECT of *N*-2-fluorenylacetamide (2-FAA) on the metabolism of a load dose of L-tryptophan was observed during studies of some of the factors involved in bladder tumorigenesis of several strains of rats fed diets containing 2-FAA and supplementary tryptophan or indole (*1*, *2*). When a challenging dose of L-tryptophan was administered to rats fed the diet containing 2-FAA, increased amounts of xanthurenic acid, its conjugates, kynurenic acid, hydroxylated kynurenines, free and

[1] Received for publication June 29, 1960.

[2] Presented in part at the Meeting of the American Chemical Society held in Miami, Florida, April 4-7, 1957. Abstract: Division of Biological Chemistry, A.C.S., No. 21, 10C, 1957.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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conjugated, and sometimes anthranilic acid glucuronide were found in the urine as compared with rats fed the basal diet without 2-FAA. No influence of the ingestion of 2-FAA upon the amount of urinary 5-hydroxyindoleacetic acid was observed.

The diets contained generous amounts of vitamin B_6, by ordinary standards, for growth and maintenance requirements. A limited increase of supplementary pyridoxine in the 2-FAA diet, however, resulted in a decrease, and in some cases prevented the appearance in the urine of detectable amounts of xanthurenic acid, kynurenic acid, and hydroxylated metabolites of kynurenine.

Since it has been reported that pyridoxine-deficient rats excrete elevated amounts of xanthurenic acid (*3*), the present findings suggest that feeding the carcinogen produces a subclinical deficiency of vitamin B_6. The possible mechanism of this effect of 2-FAA will be discussed.

MATERIALS AND METHODS

Animals and diets.—Young male and/or female rats of the Buffalo, Fischer, and A × C (Irish) strains were fed diets similar to those used by Dunning, Curtis, and Maun (*1*). At the beginning of the present investigation the basal diet had the following percentage composition: vitamin-free casein, 26; salt mixture 185a (*4*), 4; dextrin, 53; lard, 14; cod-liver oil, 0.4; choline chloride, 0.2; α-tocopherol in lard, 0.015; and cellu flour-vitamin mix, 2.0, furnishing per g of diet: B_1 hydrochloride, 4 μg; B_2, 8 μg; pyridoxine hydrochloride, 4 μg; niacin, 2 μg; and calcium pantothenate, 20 μg. Later, as a result of the tryptophan-load test, a deficiency in B_6 in rats fed 2-FAA was suspected and the concentrations of the B vitamins mentioned, including pyridoxine, were doubled in the hope that fewer of the remaining rats would die during the long feeding periods necessary for the induction of bladder tumors (*1*). Additional groups of rats were fed pyridoxine hydrochloride (referred to hereafter as pyridoxine or B_6) in dextrin pills furnishing 2000 μg of B_6 daily or it was incorporated in the diets at concentrations of 0.25, 10, 25, 80, and 100 μg per g. In a few tests pyridoxine was administered subcutaneously or intraperitoneally. One diet used contained 25 percent hydrolyzed casein and 1 percent L-tryptophan in place of 26 percent casein and another (*5*) contained 12 percent casein.

Fluorene derivatives.—2-FAA was incorporated in the diets at concentrations of 0.06, 0.04, 0.03, and 0.015 percent for different groups of rats. When administered by stomach tube in propylene glycol the dosage was 5 or 10 mg/100 g of body weight, given once only or repeated every 3d day for 6 doses. Isotopic nitrogen-labeled 2-FAA was used for recovery experiments from urine (*6*) and for protein-binding studies (*7*) by the methods previously described.[4] Tryptophan-load tests were also made

[4] Sincere appreciation is expressed to Mr. Bernard E. Burr, of the Laboratory of Physiology, National Cancer Institute, for analyses of N^{15} : N^{14} ratios in a Consolidated Nier Mass spectrometer.

on groups of rats fed *N*-(7-hydroxy-2-fluorenyl)-acetamide (7-OH-2-FAA) or *N,N*-2-fluorenyldiacetamide (2-F-diAA).

Tryptophan challenge.—Challenging doses of L-tryptophan 80 or 100 mg/rat or of 0.25 mmole (51 mg)/100 g of body weight, were first administered in dextrin pills and later, with greater accuracy, by stomach tube, usually in 2 equal doses at the beginning and at the 5th hour of the collection period. To solubilize the tryptophan, it was ground with an equal weight of dextrin and emulsified in water. The amount of L-tryptophan used in the challenge dose is critical since, within narrow limits, a dose too low does not challenge the carcinogen-fed rats or too high causes the control rats to excrete considerable amounts of xanthurenic acid. With L-tryptophan administered on a body-weight basis, the control rats in each group always received more tryptophan per rat than the somewhat smaller carcinogen-fed animals.

Although the challenges with tryptophan were made between the 1st and 12th week of experimental feeding, some rats of each group were fed continuously for much longer periods for comparison of carcinogenic effects. The final incidence and types of tumors found with the different diets and strains of rats are being reported separately (*8*, *9*).

Collection of urine.—After the load dose of tryptophan, 24-hour samples of urine of control rats on the basal diets and of rats on the basal diets containing the carcinogen were collected and brought to equal volumes as described previously (*10*). The 24-hour collection period was selected when it was found that the extra tryptophan metabolites detectable by the methods used were excreted during 24 hours after administration of the tryptophan. Hereafter, unless otherwise stated, the urines used for analyses were freshly collected, 24-hour samples from individual rats.

Methods of analysis.—The urine was analyzed for total nitrogen by a micro-Kjeldahl method (*6*). Tryptophan, anthranilic acid, and kynurenine were determined colorimetrically by the Eckert diazotization *N*-(1-naphthyl)ethylenediamine dihydrochloride-coupling method (*11*) as modified by Mason and Berg (*12*). Xanthurenic acid (XA) was measured by a slight modification of the Rosen, Lowy, and Sprince (*13*) method. The diluted urine sample was limited to a volume of 4 ml to which was added 5 ml of a carbonate buffer, *p*H 8.3, made from 0.2 percent Na_2CO_3 by saturation with 5 percent CO_2. Recovery of added XA was also determined for each sample. In experiments with Fischer strain rats identical values for XA were obtained with the method of Rosen[5] *et al.* and that of Wachstein and Gudaitis (*14*). Since the latter method was less time-consuming, it was used thereafter.

Paper chromatography.—We achieved confirmation of the spectrophotometric analyses and identification of some of the metabolites by paper chromatography, using the method of Dalgliesch (*15*) and also a modification of that procedure involving ascending, two-dimensional

[5] We wish to thank Dr. Rosen for a sample of pure xanthurenic acid made at the Ortho Company. Three different commercial samples gave approximately one third the color given by the pure material and were suspected of containing the 8-methyl ether of xanthurenic acid.

chromatography and 7 × 7 inch Whatman No. 1 papers clipped into cylinder shape (*10*).[6] Equal aliquots of one third or one half of the 24-hour samples of urine from control and 2-FAA-fed L-tryptophan-challenged rats were treated with charcoal, and the evaporated, washed phenol eluates, concentrated under reduced pressure at 38° C, were taken up in equal amounts of 0.5 or 1.0 ml of water. Comparisons were made of the metabolites present and the relative amounts of each after spotting with 10, 20, and 50 μl of each extract. On the basis of urinary nitrogen, the volumes of extracts used for the carcinogen-fed rats represented samples of urine containing less total nitrogen than those of the control rats, since the carcinogen-fed rats ingested less food daily. R_F values, fluorescence under ultraviolet light, and staining by Ehrlich and by Ekman reagents were used for identification of the metabolites (*15*). As no standards were available for conjugated metabolites, identification of glucuronides was made by comparison of chromatograms of urine samples of B_6-deficient L-tryptophan-challenged rats before and after incubation of the urine for 18 hours with bacterial β-glucuronidase (*10*). Further confirmation of xanthurenic acid conjugates has been provided by a recent report of Baglioni *et al.* (*16*).

By colorimetric methods of analysis (*12*, *13*), preliminary recovery tests were made on aqueous extracts of concentrated washed phenol eluates of charcoal used in the treatment of rat urine after addition to the urine of anthranilic acid, kynurenine, L-tryptophan, and xanthurenic acid in amounts similar to the apparent concentrations found in tryptophan-challenged 2-FAA-fed rats. The following percentages of added materials were recovered: total color-reacting, measured as tryptophan, 98; anthranilic acid, 71; kynurenine, 75; tryptophan, 114; and xanthurenic acid, 95. This high recovery of XA required elution of the charcoal with aqueous phenol, followed by elution with acetic acid-phenol, and finally with ammonia-phenol (*15*).

N^1-methylnicotinamide.—N^1-Methylnicotinamide analyses[7] were made of 28 samples of urine collected after L-tryptophan challenge to female Buffalo rats that had been maintained on the basal or the 0.06 percent 2-FAA diets of casein or of hydrolyzed casein containing 4, 10, or 100 μg of pyridoxine per g.

RESULTS

Increased Output of Metabolites of L-Tryptophan

The data in table 1 are representative of the results with the many female Buffalo rats that were tested during feeding of the casein and the hydrolyzed casein diets containing 2 to 4 μg of pyridoxine per g, with and

[6] We are grateful to Dr. Ray R. Brown of University Hospital, University of Wisconsin, for generously supplying us with samples of xanthurenic acid, xanthurenic acid-8-methyl-ether, o-aminohippuric acid, N^{α}-acetyl-L-kynurenine, zinc anthranilic acid glucuronide, and indican.

[7] We are indebted to Mr. Ernest G. McDaniel, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, for the analyses of N^1-methylnicotinamide (*17*).

TABLE 1.—Comparison of tryptophan metabolites in urine of individual control and *N*-2-fluorenylacetamide-fed female Buffalo rats after administration of L-tryptophan*

Diet†	Total color-reacting material measured as tryptophan‡		Reacting as kynurenine‡		Reacting as anthranilic acid‡		Reacting as xanthurenic acid§	
	mg	μg per mg of urinary nitrogen	mg	μg per mg of urinary nitrogen	mg	μg per mg of urinary nitrogen	mg	μg per mg of urinary nitrogen
Basal casein‖	4.7	35	0.5	3.7	0.4	3	1.5	11
Basal casein + 0.06 percent 2-FAA‖	15.4	173	6.7	75.0	1.4	16	5.3	59
Basal hydrolyzed casein + L-T¶	3.2	22	0.5	3.5	0.3	2	2.2	15
Basal hydrolyzed casein + L-T + 0.06 percent 2-FAA¶	5.8	49	2.5	21.0	0.6	5	7.8	65

*100 Mg of L-tryptophan in a 5.0 percent aqueous suspension was administered to each rat at the beginning of the 24-hour period of urine collection.
†40th Day on diets.
‡Eckert-Mason, Berg diazotization-coupling method.
§Rosen, Lowy, and Sprince iron reaction method.
‖26 Percent casein and 4 μg pyridoxine per g of diet.
¶25 Percent hydrolyzed casein + 1 percent L-tryptophan (L-T) and 4 μg of pyridoxine per g of diet.

without 0.06 percent 2-FAA. Three to 4 times more XA appeared in the urine of carcinogen-fed rats than in control animals. Total color-reacting material, anthranilic acid glucuronide, and kynurenine derivatives were excreted in larger amounts by the 2-FAA-fed rats than by rats fed the respective basal diets. In the absence of a load dose of tryptophan neither quantitative nor qualitative differences in tryptophan metabolites were detected in the urine of rats maintained on these diets with and without the carcinogen. Moreover, when a single dose of 5 or 10 mg of 2-FAA per 100 g of body weight was given by stomach tube to rats maintained on the basal diet and the L-tryptophan load was administered intraperitoneally, subcutaneously, or by stomach tube, simultaneously, or 6 or 24 hours after the carcinogen, no effect of 2-FAA on urinary tryptophan metabolites was observed.

The amount of XA excreted by the same rat after a challenge dose of L-tryptophan varied at different times, but there was no evidence of an increase with time after the first few weeks of feeding the carcinogen. In some rats the effect was less at 12 than at 4 weeks. The effect, moreover, was observed when the livers of animals killed at the time of the test showed no macroscopic evidence of damage. Histologic studies were not made.

The influence of 2-FAA on load-tryptophan metabolism was most pronounced in female Buffalo rats when the concentration of 2-FAA in the diet was 0.06 percent and the concentration of pyridoxine was not greater than 2 μg per g. However, with 0.06 percent 2-FAA in the diet, the effect on tryptophan metabolism was demonstrable in rats maintained on 2, 4, or 8 μg of pyridoxine per g of food. With the diet containing 4 μg pyridoxine per g the effect was less with the carcinogen at 0.03 percent, and was not manifested in female rats when 0.015 percent 2-FAA was fed. Male rats are more susceptible to the toxic effects of 2-FAA, and showed a greater interference than female rats in the metabolism of a load dose of tryptophan, with a concentration of 0.04 or 0.03 percent 2-FAA in the diet.

When a challenge dose of D-tryptophan was administered at the same level and under the same experimental conditions as L-tryptophan, no XA was detected in the urine.

Possible Role of Vitamin B_6

When pyridoxine-dextrin pills furnishing 100 μg of B_6 daily were fed to some of the rats for 2 days preceding and during the 24-hour collection period after the challenge with L-tryptophan, XA, kynurenic acid, and hydroxylated kynurenine, metabolites were reduced in quantity. As seen in table 2, in groups of rats fed diets with 8 μg of pyridoxine per g more XA was excreted by rats on the carcinogenic diet than by those on the basal diet. With the diet containing 2-FAA and 80 μg of pyridoxine, less XA appeared in the urine after the L-tryptophan challenge than with 2-FAA and 8 μg of pyridoxine per g.

Four groups of weanling female Buffalo rats, fed the same basal and 0.06 percent 2-FAA-casein diets containing supplementary pyridoxine to make 10 and 100 μg B_6 per g of food, were challenged with tryptophan biweekly for 8 weeks. The rats maintained on the diet containing 100 μg of B_6 and 2-FAA frequently excreted more XA and other tryptophan metabolites in response to the challenge than rats fed the diet containing 10 μg of pyridoxine per g. To explore these observations further, dextrin pills, providing 2000 μg of pyridoxine daily, were fed to the rats for a week preceding and during a challenge test. This large supplement of B_6 also appeared to have a stimulating effect on the excretion of XA by the rats maintained on the basal diet with or without 2-FAA.

In another comparison male and female rats fed for 2 months on a B_6-deficient (0.25 μg pyridoxine/g) *basal* diet excreted as much XA

TABLE 2.—Effect of a tenfold increase in the B_6 content of the diet of female Buffalo rats on the amount of xanthurenic acid excreted after administration of L-tryptophan

Time on diet (weeks)	Diet	8 μg Pyridoxine/g of diet		80 μg Pyridoxine/g of diet	
		Tryptophan administered* (mg)	Xanthurenic acid excreted† (mg)	Tryptophan administered* (mg)	Xanthurenic acid excreted† (mg)
1. 0	Basal casein	102	0	—	—
3. 0	"	102	0. 4	102	0. 6
3. 5	"	—	—	102	0. 5
3. 5	"	102	0. 3	110	0. 4
11. 0	"	102	0. 4	114	1. 3
11. 0	"	135	0. 9	108	0. 6
1. 0	Basal casein + 0.06 percent 2-FAA	102	3. 4	—	—
3. 0	"	102	3. 2	102	0. 3
3. 5	"	102	2. 0	102	1. 3
11. 0	"	69	4. 4	86	1. 5
11. 0	"	102	4. 6	86	2. 7

*L-tryptophan was administered in 2 doses at the 0 and 5th hour of the 24 hours of urine collection.
†Xanthurenic acid of urine was determined by the colorimetric method of Rosen, Lowy, and Sprince.

after being challenged with tryptophan as rats maintained on 0.06 percent 2-FAA diets containing 4 or 8 μg of pyridoxine per g. The challenging dose of tryptophan to the rats on the B_6-deficient diet was therefore reduced. Table 3 shows that the urinary output of XA was greater from both male and female rats fed the B_6-deficient diet containing 0.03 percent 2-FAA after half the load dose of L-tryptophan than from those animals fed the basal diets. The effect of 0.03 percent 2-FAA was much greater with male than with female rats. Thus when the diet was supplemented with 25 μg per g and twice as much tryptophan was used for the challenge, the excretion of XA was greater from male animals receiving 0.03 percent 2-FAA than on the basal diet, but no effect of 2-FAA was observed with female rats on the same high B_6 regimen.

TABLE 3.—Comparison of urinary xanthurenic acid output after a challenge dose of L-tryptophan to Buffalo strain rats on deficient- and high-vitamin B_6 diets*

Diet	24-Hour urinary output of xanthurenic acid			
	Males		Females	
	0.25 μg Pyridoxine/g of diet (mg)	25.0 μg Pyridoxine/g of diet (mg)	0.25 μg Pyridoxine/g of diet (mg)	25.0 μg Pyridoxine/g of diet (mg)
Basal	3.0	0.8	10.0	2.0
	3.7	1.6	3.2	1.4
	—	1.2	—	—
Basal + 0.03% 2-FAA	23.0	2.7	14.0	2.0
	8.4	12.4	6.7	1.1
	24.0	4.5	—	—

*Challenge of 0.125 millimoles L-tryptophan per 100 g of body weight to rats fed B_6-deficient diets and of 0.25 millimoles per 100 g of body weight with high B_6 diets.

Effect of Low and High B_6 Intakes on 2-FAA

No difference was found in the urinary excretion of groups of metabolites of 2-FAA by rats on the B_6-deficient and on the high B_6 diets. Ether-soluble metabolites of the urine at *p*H 6 accounted for 9 to 14 percent of the total N^{15} recovered; ether-soluble metabolites after incubation of the urine with bacterial β-glucuronidase, 52 to 56 percent; and water soluble N^{15} material after the 2 preceding ether extractions, 33 to 37 percent. Colorimetric tests made on these same urine samples and on the urine of other rats on these diets containing 0.03 percent 2-FAA indicated that there was no significant difference in total diazotizable-R-salt-reacting material, in nitrite-reacting (hydroxylated metabolites), or in the proportion of free amines present in the urine of the rats fed the low and high pyridoxine diets.

No differences were observed in the binding of N^{15} from 2-FAA-N^{15} by male Buffalo or Fischer strain rats maintained on the basal B_6-deficient and those fed the diet containing 25 μg of pyridoxine per g. The concentrations of isotopic nitrogen in the liver proteins of control rats administered repeated doses of unlabeled 2-FAA were 0.0065 ± 0.0005 atom percent excess N^{15} for the coagulated soluble proteins and 0.0060 ± 0.0005 atom percent excess N^{15} for the insoluble protein fractions, while the N^{15} concentrations of the protein fractions from rats administered 2-FAA-N^{15} were 0.0096 to 0.012, and 0.008 to 0.010, respectively. Binding of N^{15} was considerably less by liver protein fractions from male rats fed continuously the high and low B_6 diets containing 0.03 percent 2-FAA-N^{15}, an observation made previously in other studies (7).

Effect in Rats of the Fischer Strain

Male and female rats of the Fischer strain showed an effect of 2-FAA similar to those of the Buffalo strain, and excreted more XA and kynurenine derivatives after a challenging dose of L-tryptophan than control rats on the basal diet. Table 4 presents the data for urinary XA excretion.

TABLE 4.—Effect of *N*-2-fluorenylacetamide on urinary output of xanthurenic acid by Fischer-strain rats after L-tryptophan*

Diet†	Dose of L-tryptophan per 100 g of body weight (millimoles)	Time of test (weeks)‡	Xanthurenic acid excreted in 24 hours (mg)
		Females	
Basal	0.3	2	0.7
"	"	2	0.8
Basal + 0.06% 2-FAA	0.3	2	2.7
"	"	3.3	2.2
"	"	7.0	1.6
"	"	8.0	3.3
"	0.4	5.0	4.1
		Males	
Basal	0.25	6.2	1.2
"	0.30	2.0	3.0
"	"	3.6	0.5
Basal + 0.06% 2-FAA	0.3	2.0	11.7
"	"	2.6	6.1
Basal + 0.04% 2-FAA	0.25	2.1	6.4
"	"	4.3	4.3
"	0.30	3.3	10.1

*L-Tryptophan in dextrin solution administered by stomach tube in 2 doses at 0 and 5th hour of 24-hour collection period.

†These diets contained 2 μg of pyridoxine per g.

‡Interval between beginning of experimental feeding and test with tryptophan.

N,N-2-Fluorenyldiacetamide

This very active hepatocarcinogen, for A × C strain male rats maintained on the 12 percent casein diet containing 2 μg of pyridoxine per g (*5*), led to an increased excretion of XA, free and conjugated, kynurenine derivatives, and anthranilic acid glucuronide after a tryptophan load, as compared with control rats maintained on the basal diet. The L-tryptophan-challenged control rats fed this basal diet also excreted considerable amounts of the materials reacting in the Mason-Berg (*12*) method as anthranilic acid and kynurenine, but no colorimetrically detectable XA.

After the tryptophan challenge no significant difference was found in the increase in urinary output of XA by Buffalo female rats maintained on the 26 percent casein-8 μg of pyridoxine per g basal diet containing either *N,N*-2-fluorenyldiacetamide or *N*-2-fluorenylacetamide. Unlike the results with the monoacetyl derivative, however, rats fed the diet containing the diacetyl compound and supplemented with 80 μg of pyridoxine per g excreted as much XA as those fed 8 μg.

N-7-Hydroxy-2-Fluorenyl-Acetamide

In this experiment no XA was detected in the urine after total doses of 95 to 102 mg of L-tryptophan were given to male Buffalo rats maintained for 13 days on the basal 26 percent casein diet containing 2 μg of pyridoxine per g. At this time the rats fed the diet containing 0.04 percent 2-FAA excreted 3.6 to 4.9 mg of XA after doses of L-tryptophan varying from 71 to 92 mg, whereas those rats fed the same basal diet containing 0.085 percent 7-OH-2-FAA (twice the molecular equivalent of 0.04 percent 2-FAA) excreted no detectable to trace amounts of XA following challenges with 97 to 102 mg L-tryptophan. After 30 days on the diets, still no XA was found in the urine of tryptophan-challenged rats maintained on the basal diet or of those on the 7-OH-2-FAA diet, while one surviving rat maintained on the 2-FAA diet excreted 8.7 mg of XA.

Paper Chromatography

Under our experimental conditions no hydroxylated derivatives of kynurenine (K), no kynurenic acid (KA), and only traces of XA were found in chromatograms of tryptophan-challenged control rats maintained on diets containing 4 to 8 μg of pyridoxine per g. Tryptophan-challenged 2-FAA-fed rats showing a moderate effect of the carcinogen contained increased amounts of K, XA, KA, XA glucuronide, and two other conjugates of XA. The maximum influence of 2-FAA feeding on the metabolism of a tryptophan load was manifested in a greater urinary output of K, KA, XA, and XA conjugates in addition to an increase in anthranilic acid glucuronide, and the appearance of *N*-acetylkynurenine, *N*-acetyl-3-hydroxykynurenine, 3-hydroxykynurenine glucuronide, and 3-hydroxykynurenine sulfate.

The chromatograms demonstrated that while materials were measured spectrophotometrically as kynurenine, anthranilic acid, tryptophan, and xanthurenic acid, the urine actually contained kynurenic acid, kynurenine and conjugates of hydroxylated kynurenine, and free and conjugated xanthurenic acid. No free anthranilic acid was present. Tryptophan, moreover, was not detected except in the urine of L-tryptophan-challenged male rats on diets containing 0.25 μg pyridoxine per g. In spite of the limitations of the spectrophotometric methods in the presence of a mixture of metabolites and conjugates of metabolites, the relative quantities estimated with these methods were essentially confirmed by paper chromatography for each of the experimental conditions, diets, and compounds described.

N^1-Methylnicotinamide

Although the urine samples from tryptophan-challenged rats fed the casein or the hydrolyzed casein diets containing the carcinogen with 4 or with 100 μg of pyridoxine per g contained relatively large amounts of XA compared with rats fed the same diets containing 10 μg pyridoxine, or

fed the basal diets without any carcinogen, no differences relative to the pyridoxine intake or the XA output were found in the amounts of N^1-methylnicotinamide in aliquots of these samples of urine. The urinary N^1-methylnicotinamide varied from 2.0 to 4.8 μmoles (average 3.2 ± 0.1). These values are not unusual for tryptophan-challenged normal rats, which have been found to excrete larger amounts of the nicotinamide metabolite than rats not administered the amino acid (*18*).

DISCUSSION

Evidence has been presented to support the conclusion that *N*-2-fluorenylacetamide in the diets of rats results in an increased urinary output of free, and/or conjugated xanthurenic acid, kynurenic acid, and hydroxylated metabolites of kynurenine after administration of a challenging dose of L-tryptophan. The magnitude of the effect is dependent upon the concentration of 2-FAA in the diet, the pyridoxine content, and possibly, to some extent, upon other unidentified factors in different diets. Moreover, there are limits to the size of the tryptophan load that will show the effect.

The chromatogram of Baglioni *et al.* (*16*) of 1/20th the 24-hour sample of urine of a normal rat after ingestion of 100 mg of L-tryptophan per 100 g of body weight resembles our chromatograms of 1/100th to 1/200th the 24-hour urine sample of rats that had been maintained on 2-FAA and 4 to 8 μg of pyridoxine per g of diet and had received 51 mg L-tryptophan per 100 g of body weight by stomach tube. Baglioni *et al.* fed a diet containing 100 μg pyridoxine per g, an amount that we frequently found to be associated with an increase in the amounts of tryptophan metabolites excreted, even by challenged normal control rats. Moreover, we found also that control rats excreted large amounts of tryptophan metabolites when challenged with 102 mg of L-tryptophan per 100 g of body weight.

Since the livers of Buffalo male rats are more susceptible to tumor induction by 2-FAA (*9*, *19*) than those of females, it is interesting that the males excreted more XA, KA, and other hydroxylated metabolites of kynurenine than females in response to an equal load of tryptophan and a lower concentration of the carcinogen in the diet. The possibility that hepatocarcinogenic activity might be associated in some way with the action of the carcinogen responsible for its effect on the metabolism of load tryptophan was supported also by the failure to find any influence on XA excretion of the hepatononcarcinogenic (*20–22*) 7-hydroxy derivative of 2-FAA even at twice the molar level, compared with 2-FAA. However, the lack of effect of 7-OH-2-FAA may be due to the little metabolism, other than conjugation, that this compound appears to undergo in the rat (*23*, *24*). The *N*,*N*-diacetyl derivative of 2-fluorenamine fed to A × C male rats at a concentration that induces liver tumors in a relatively short time had, moreover, approximately the same amount of effect on load-tryptophan metabolism as 2-FAA fed to Buffalo male rats even though the tumor induction period in the latter was considerably longer.

The likelihood that the increased urinary output of XA might be due to a 2-FAA-produced deficiency in B_6 (*3*) was of interest in view of some earlier observations reported in the literature. Miner, Miller, Baumann, and Rusch (*25*), and Miller, Baumann, and Rusch (*26*) found that a low dietary B_6, independent of caloric intake, reduces the incidence of liver tumor induced in rats by *p*-dimethylaminoazobenzene. Harris (*27*, *28*) reported that the incidence of liver tumors was lower in rats of both sexes fed 0.04 percent 2-FAA in a diet containing 0.2 μg of pyridoxine per g than in those fed similar diets containing 20 μg of pyridoxine per g. Buffalo rats, in this laboratory, fed a diet containing 0.03 percent 2-FAA and 25 μg of pyridoxine per g and killed at 1-, 2-, and 3-month intervals, developed earlier and more extensive pathologic changes in the livers of both sexes, including hepatomas in males, than rats fed the same diet containing 0.25 μg of pyridoxine per g (*9*). Thus there is evidence that vitamin B_6 promotes hepatocarcinogenesis induced by 2 amino carcinogens.

In the absence of a tryptophan challenge, there was little evidence to suggest that the effect of vitamin B_6 on tumor induction is due to a role of the vitamin in the metabolism of the carcinogen. It is possible that the slower appearance of liver tumors in the B_6-deficient rats (*9*, *25–27*) is related to a poorer state of nutrition in those animals as a result of the low pyridoxine content of the diet, a possibility supported by the observations of Kline *et al.* (*29*). On the basis of total food consumption, unrelated to body weight, the rats of both sexes ingested a little more food and 2-FAA on the high than on the low B_6 diet during the precancerous period, *i.e.*, 12 weeks.

If a direct effect of 2-FAA upon pyridoxine phosphate *is* involved, it is not apparent in the way that L-penicillamine exhibits an effect on the vitamin-B_6 requirement of the rat by inhibition of growth and production of acute, severe symptoms of the vitamin deficiency followed by death, with the symptoms readily overcome by the injection of 1 mg daily or by dietary pyridoxine supplements (*30*). In our experience no clinical symptoms suggestive of B_6 deficiency were seen. Both the high and the low B_6 diets containing 0.03 percent 2-FAA, when fed to young adult rats, inhibited further increases in body weight of both sexes (*8*).

That a small supplement of pyridoxine, added to the food or administered at the time of the tryptophan challenge, will lessen the effect of 2-FAA upon the urinary XA output suggests, however, that 2-FAA may compete with some of the metabolites of L-tryptophan for pyridoxal phosphate (PP). Jakoby and Bonner (*31*) reported that kynureninase obtained from *Neurospora crassa* is inhibited by 28 amine compounds tested. They found, furthermore, that amine inhibition was due in most cases to the removal of the coenzyme, PP, and it was suggested that the amine and PP are combined in the form of a Schiff base. PP activated L-kynurenine, 3-hydroxy-L-kynurenine, and N^1-formyl-L-kynurenine for their metabolism in the presence of kynureninase, to alanine and anthranilic acid, 3-hydroxyanthranilic acid, and formylanthranilic acid, respectively (*32*). Since 2–FAA is readily deacetylated in the rat, it is possible that the free

amine or the free amine of a metabolite of the carcinogen reacts with PP and thus creates a deficiency in the concentration of the free coenzyme.

Administration of a large dose of L-tryptophan induces an increase in the peroxidase-oxidase (pyrrolase) system that catalyzes the conversion of L-tryptophan to formylkynurenine (*33*). It is generally accepted that there is a great excess of formylase in rat liver. Thus a rapid accumulation of kynurenine may be expected to occur after the challenging dose of tryptophan. If kynureninase in the rat behaves like that from *Neurospora*, such a reaction between an amine metabolite of 2-FAA with PP may be responsible for the accumulation of the kynurenine derivatives. In no case was anthranilic acid or 3-hydroxyanthranilic acid detected in the urine. Anthranilic acid glucuronide, however, was invariably present in small amounts in the urine of control rats after a tryptophan challenge and it was found in increased amounts in the urine of those L-tryptophan-challenged 2-FAA-fed rats that excreted the largest amounts of xanthurenic acid. Since no evidence was found of a decrease in urinary N^1-methylnicotinamide in the urine of L-tryptophan-challenged 2-FAA-fed rats that excreted large amounts of XA, it must be concluded that some of the kynurenine was metabolized to nicotinamide. The accumulation of XA and KA in the urine of tryptophan-challenged B_6-deficient rats suggests that there must be a difference in the mechanisms of the coenzyme activity of PP with kynureninase, which is *inhibited* by B_6 deficiency, and with the PP-dependent transaminase which is believed to catalyze the production of XA and KA from 3-hydroxykynurenine and kynurenine respectively (*31*).

The finding that 50 times or more the usually accepted dietary requirement of B_6 had no inhibiting effect, but actually a stimulating effect upon the excretion of XA, may be explained if the behavior of PP is similar to that observed in the activity of kynureninase from *Neurospora* (*31*). In this system inhibition of kynurenine by amines is reversed by the addition of PP. PP in excess, however, is inhibitory. The experimental results indicated that the inhibition by PP was due more to the formation of an analogue type of inhibitor composed of the Schiff base of kynurenine and the coenzyme rather than to removal of kynurenine or PP by the Schiff base reaction (*31*).

Kynurenine, the substrate of the enzyme, was found to act as its own inhibitor when present in high concentrations (*31*). The mechanism of the inhibitory effect of high kynurenine concentrations was postulated to be due, in part at least, to the removal of PP as a kynurenine PP complex. In the rat the tryptophan challenge may cause a decrease in available PP through the accumulation of L-kynurenine. This decrease in free coenzyme as a result of a reaction with high concentrations of kynurenine, aggravated by the decrease in available PP caused by its reaction with 2-fluorenamine, would account for the greater accumulation and excretion of intermediate metabolites of L-tryptophan in the 2-FAA-fed animals than in the controls fed the basal diet.

The observations described here are part of a study undertaken with

the hope of obtaining information on the mechanism of carcinogenesis of 2-FAA. A relatively low concentration of dietary B_6 has been associated with an increase in the period of induction and/or a lower incidence of tumors of the liver by 2-FAA and by an azo-dye carcinogen. Thus the action of 2-FAA in reducing B_6 availability in the rat would tend to retard rather than promote its hepatocarcinogenic activity. These findings may explain the requirement of the rat for an ample supply of vitamin B_6 during the induction of liver tumors by aromatic amino carcinogens.

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Polyoma Virus and Production of Malignancy *In Vitro*[1]

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SUMMARY

An attempt was made to induce a rapid and reproducible neoplastic conversion in a single-cell clone of mouse cells during the period *in vitro* before "spontaneous" conversions occur. Such changes were repeatedly produced in a clone derived from the salivary gland of a C3H mouse by infection with polyoma virus. The mechanism of this change, whether by cell selection or by cell change, awaits further study. Cells of the salivary-gland clone were also compared with 2 other long-term strains of C3H mouse cells, one derived from minced whole embryos and the other from adult connective tissue. Cells were compared with respect to viral susceptibility and tumor-producing capacity. Of the 3 cell types, only the adult fibroblasts from connective tissue failed to show any cytopathogenic response to infection with polyoma. It was shown further that all 3 cell types cultured in a serum-supplemented, chemically defined medium will ultimately undergo a malignant conversion *in vitro* when no virus is purposely added. Tumors produced by the salivary-gland clone were extraordinary because of the amount of collagen they contained and the distortion of the leg they produced when cells were transplanted to the thigh muscles.—J. Nat. Cancer Inst. 26: 331–357, 1961.

AS EARLY as 1925, Carrel wrote that "the best method of ascertaining the properties that characterize a malignant tissue would be to transform *in vitro* a strain of cells of a known type into cells capable of producing sarcoma or carcinoma, and to study the changes undergone by the strain. The comparison of the normal and the malignant strain would show what characteristics are specific of the state of malignancy" (*1*). Since that time, numerous attempts have been made to attain this objective. Frequent reports of a controlled "cancerization" of normal tissue cells in culture by the direct action of carcinogenic agents have appeared in the literature.

The earliest of these reports by Fischer (*2*), Laser (*3*), Bisceglie (*4*), and des Ligneris (*5*, *6*) described the transformation of chicken-embryo cells by the use of dilute arsenous acid, tar, plasma from chickens receiv-

[1] Received for publication August 3, 1960.

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ing injections of tar, X radiation, and dibenzanthracene, respectively. The tumors produced from inoculated cultures resembled, in most cases, the Rous sarcoma and were transmissible by cell-free filtrates. These experimental results could not be confirmed by others (*7*, *8*) and for several reasons, as reviewed by Begg and Cramer (*9*), Knake (*10*), and Larionow (*11*), appear to have resulted from accidental infection of the cultures with chicken-tumor viruses.

The first convincing evidence of a malignant conversion of normal tissue cells *in vitro* was presented by Gey (*12*) and Earle (*13*, *14*) in 1941 and 1943, respectively. Gey and associates (*15*) reported 3 apparently spontaneous conversions in a strain of rat fibroblasts cultured from areolar connective tissue, and Earle studied in detail such transformations in carcinogen-treated and one untreated strain of C3H mouse cells cultured from subcutaneous adipose tissue. Further studies by Sanford and associates (*16*, *17*) showed that under certain culture conditions such conversions may occur regularly in the absence of any known carcinogen in cells from connective tissue or skeletal muscle of strain C3H mice, some bearing the milk agent and some agent-free. However, a variable but long period of growth in culture appeared to precede the malignant change. In other reports of malignant conversions of mouse and rat cells *in vitro* by Benevolenskaya (*18*), Mogila (*19*), and Goldblatt and Cameron (*20*), specific carcinogenic agents such as methylcholanthrene, the milk agent, tumor filtrates, or intermittent exposure of cultures to anaerobiosis were considered the causative factors. However, in the first two experimental studies no control cultures were tested *in vivo* for neoplastic potentialities, and in the third study only one control line of cells was tested. In Goldblatt and Cameron's experiments, the cultures were exposed to a streaming of pure nitrogen which, as pointed out by Harris (*21*), probably caused a marked rise in *p*H and made it difficult to be certain that the injury to the cells was due to anoxia alone.

The first attempt to transform normal cells *in vitro* with a tumor virus was reported by Carrel (*22*) who treated cultures of blood monocytes with a cell-free filtrate of Rous sarcoma and then cultured the cells for several weeks before inoculation into chickens. The inoculated cultures always produced tumors, but so did the supernatant culture fluids. In evaluating these experiments, Lewis (*23*) raised a question as to whether the tumors were produced by the implanted monocytes or by the virus associated with the cells. Ludford (*24*) carefully reviewed the experiments of Carrel, but produced evidence that the fibroblast rather than the monocyte is the cell susceptible to infection with the Rous virus. This conclusion was confirmed by experiments in this laboratory (*25*). However, capacity to support a tumor virus in its "infectious" state is not a criterion of malignancy (*26*). Nor, at the present stage of our knowledge, can morphologic alterations resembling certain cancer cells or increased growth activity in culture be considered reliable indexes of neoplastic change, though several workers have reported cancerization of cells *in vitro* on the basis of these criteria alone (*27*, *28*).

A malignant tumor is generally defined as an autonomous, progressively growing neoplasm. Until some property of cells in culture can be definitely correlated with this capacity, only one test for malignant change in tissue-culture cells is available. This test is to assay the cells for their capacity to grow autonomously and progressively when reimplanted into animals of the strain of origin. In the application of this test to cells infected with tumor viruses, it is essential to know that the resultant tumor arises from the implanted cells and not from the virus-infected host tissues. Although inoculations of the infected cells may be compared with those of cell-free virus, any differences in tumor production or tumor latent periods may result from the reduced viral activity of filtered or cell-free virus as compared with activity of virus liberated continuously from living cells. Most reports of neoplastic or malignant change induced by viruses in culture are uncertain because of failure or inability to identify the tumors produced by implantation of the virus-infected cells (*29*). One exception is the report by Coman (*30*) who inoculated rabbit epidermis in culture with Shope papilloma virus. Virus-treated and untreated cultures, as well as virus alone, were implanted in the liver of the same rabbit from which the epidermis was derived. Benign tumors developed, but only from the virus-treated cultures. The tumor cells could be identified morphologically as squamous epithelial cells distinctly different from the liver cells. Malignant conversion was not produced.

For biochemical studies of the events associated with a neoplastic transformation of cells *in vitro*, a reproducible, rapid conversion in massive cultures of cells, preferably of single-cell origin and grown in chemically defined medium, would be desirable. Massive cultures would probably be necessary since most chemical determinations, especially of enzyme activities, require large numbers of cells. Since cells can undergo apparently spontaneous conversions after prolonged culture *in vitro*, the experiments would, of necessity, have to be limited to the early months of culture.

In the study reported here, an effort was made to induce a controlled malignant conversion *in vitro* in a clone of mouse cells by means of the polyoma virus. The polyoma virus was selected as the carcinogenic agent, since this virus produces autonomous neoplasms, particularly parotid-gland tumors, in mice of inbred strains such as C3H (*31–33*). Cells from inbred animals provide suitable genetic material for assay of neoplastic potentialities *in vivo*. Further, cells from a strain C3H mouse could be transplanted to F_1 hybrid mice such as (C3H $\times$ BALB/c)F_1. If a tumor arose in the hybrid host, it would be possible by transplantation tests to establish whether the tumor originated from the implanted cells or from the host tissues (*34*). Therefore, in this study a clone of cells was established in culture from the normal salivary gland of a C3H mouse, and cultures treated with polyoma virus as well as untreated control cells were assayed for neoplastic potentialities in hybrid hosts. Cells of the salivary-gland clone were also compared with two other

long-term strains of C3H mouse cells, one derived from minced embryos and the other from subcutaneous connective tissue of an adult mouse. These cell strains were compared with respect to viral susceptibility and tumor-producing capacity when grown under similar culture conditions in a serum-supplemented, chemically defined medium.

METHODS

Establishment of cell strains and clone.—Three cell strains and one clone were established in culture. The first cell strain, NCTC 2661, was originated in January, 1957, from areolar and adipose connective tissue taken from the subcutaneous fat pad in the left flank of an agent-free, 82-day-old male mouse of strain C3Hf/He. The explant of tissue was inserted under a sheet of perforated cellophane (*35*) in a Carrel D-3.5 flask containing 1 ml of culture medium. The culture medium used, except as indicated, was NCTC 109 supplemented with horse serum to a final concentration of 10 percent (*36*, *37*). No antibiotics were used. The cultures were gassed with a humidified mixture of 5 percent CO_2 in air to adjust the *p*H of the medium, were sealed with silicone stoppers, and incubated at 37.5° C. The culture medium was renewed 3 times a week, at which time each culture was gassed as described.

The cells of strain 2661 grew slowly as fibroblasts and were not transplanted for the first 19 weeks. We transplanted cells by gently loosening the growth from the cellophane sheet on the floor of the flask by means of a platinum-iridium needle (*38*). The suspension of cells in culture fluid was then transferred by pipette to 2 new flasks. After the first few months *in vitro*, the cells were transplanted approximately every 2 weeks to Carrel D-3.5 or T-60 flasks (*39*) with or without cellophane substrate. Frequently the parent culture containing dislodged cells was also retained and allowed to repopulate. In the tabulations of this study the repopulated parent culture was considered to be of the same transplant generation as the 2 daughter cultures.

The second cell strain, NCTC 2881, was originated in October, 1957, from a mince of mouse embryos of strain C3H/HeN. The embryos (approximately 9 mm long) were washed in the culture fluid, minced with scissors, and passed through a circular piece of 24-mesh, stainless steel gauze fitted in the bottom of a 50 ml Luer syringe. The resultant pulp was received in a 100 ml centrifuge tube, diluted with culture medium, and aliquots of the cell suspension were used to inoculate 3 T-60 flasks. Cells were cultured on the glass floor of the flask, according to procedures described, and were not transplanted for the first 9 weeks. After a few months *in vitro*, the cells resembled fibroblasts and 1 culture was transplanted to 2 flasks approximately every 2 weeks.

The third cell strain was originated in November, 1958, from parotid and submaxillary tissue taken from a 65-day-old male mouse of strain

C3H/HeN. The procedures for culture were similar to those described for cell strain 2661. However, the cells formed a more flattened epithelium-like cell sheet. Cells of the first generation were transplanted after 9 weeks; thereafter, cells were transplanted at approximately 2- or 3-week intervals.

In May, 1959, one cell was isolated from a culture of the ninth transplant generation by a simplified capillary-cloning technique (*40*). As the isolated single cell proliferated, the progeny migrated out the open ends of the capillary onto the floor of the flask to establish the clone, NCTC 3435 (fig. 1). Cells of this clone formed epithelium-like cell sheets, but also at times showed a fibroblast-like morphology (figs. 4 and 5).

Photomicrographic procedures.—Living cultures were photographed by procedures described (*41*). Cells were photographed 24 hours after a renewal of culture fluid.

Injections of culture cells into mice.—The procedures for injection of culture cells into the left thigh muscles of mice have been described (*16, 42*). Each mouse received the cells suspended in 1 ml culture fluid, except as indicated. Two groups of mice, as noted later, were less than 24 hours old. Each of these received an injection of approximately 0.2 ml of concentrated cell suspension by the insertion of a 27-gauge needle near the tail and along the middorsal line to the neck and head, where the cell suspension was delivered subcutaneously.

The adult mice used were 1- to 5-month-old males of strains C3H/HeN, BALB/cAnN (referred to herein as C) and the hybrid (C3H $\times$ C)F_1. The hybrid mice were used only for assay of clone 3435 culture cells. All mice inoculated with culture cells were irradiated with a whole-body dose of 425 r several hours before inoculation to lower their resistance (*43*).[4] Physical factors were 200 kv, 15 ma, 120 r/minute at a focal distance of 54 cm through 0.25 mm Cu and 0.55 mm Al filters. Mice used for subinoculation of tumors were not irradiated.

Mice were observed for 3 to 4 months after inoculation. The site of inoculation was palpated weekly to determine the latent period for tumor development. The latent period measured the interval from injection of cells to the appearance of a palpable tumor at the site of injection. Autopsies were performed on all mice; sections of tumor tissue were fixed in Zenker-formol and stained with hematoxylin and eosin and with van Gieson's picrofuchsin.

Replicate culture procedures.—Replicate cultures were prepared from sieved, continuously stirred suspensions of cells in culture fluid. These quantitative procedures including the method for renewal of culture fluid have been described (*38*). Numbers of cell nuclei, used as an estimate of cell numbers, were determined by the nuclei enumeration procedure (*44*).

Polyoma virus infection of cultures.—The LID-1 strain of polyoma virus (*45*) was kindly supplied by Dr. Wallace Rowe of the National Institute of Allergy and Infectious Diseases. A pool of the seventh and eighth

[4] The authors express their appreciation to Mr. Henry Meyer of the Radiation Branch of this Institute, who irradiated the mice.

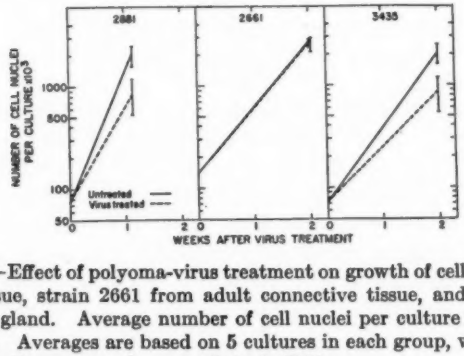
passages in mouse-embryo cultures was harvested between April and June, 1958, and two vials were obtained from Dr. Rowe in May and October, 1959. The virus suspension was diluted to 10^{-2} with NCTC 109 supplemented with 2 percent heated horse serum (56° C for 30 min.), passed through a Selas #03 filter, and stored at −50 to −60° C before use. This preparation was used in the first 2 experiments. In the third experiment, the virus was recovered from infected cultures of the second experiment. Used culture fluids were collected 48 hours after a fluid renewal and, 11 days after infection, were pooled and passed through a Selas #03 filter. Control medium from the untreated cultures was similarly prepared.

In the first and third experiments, 1 ml of virus suspension or 1 ml of control medium NCTC 109 with 2 percent heated horse serum was added to each T-15 replicate culture containing 0.5 ml of cell suspension and 1.5 ml of culture fluid. In the second experiment 4 ml of virus suspension or 4 ml of control medium was added to each T-60 replicate culture containing 2 ml of cell suspension and 4 ml of culture fluid.

RESULTS

Effect of Polyoma on Cells in Culture

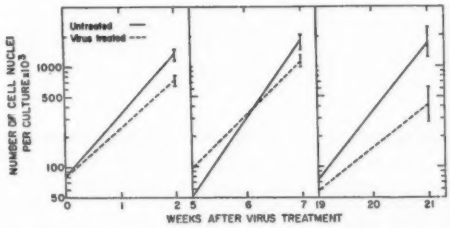
The effects of polyoma-virus treatment on cells of strain 2881 from embryonic tissue, strain 2661 from adult connective tissue, and clone 3435 from adult salivary gland were compared. In the first quantitative experiment, a cytopathogenic effect, characterized by localized necrosis and detachment of cells, was observed by 7 to 10 days in treated cultures of strain 2881 and clone 3435 (figs. 2 and 3) but not at any time in cultures of strain 2661. Determination of cell numbers in replicate cultures at 8 and 14 days after treatment likewise revealed a significant difference in cell numbers between virus-treated and untreated cultures of 2881 and 3435, but no difference in cultures of 2661 (text-fig. 1). Three additional experiments with different inoculum sizes confirmed these observations



TEXT-FIGURE 1.—Effect of polyoma-virus treatment on growth of cell strains 2881 from embryonic tissue, strain 2661 from adult connective tissue, and clone 3435 from adult salivary gland. Average number of cell nuclei per culture is plotted against time in weeks. Averages are based on 5 cultures in each group, with the exception of 3 cultures in the untreated group of strain 2661.

(figs. 6 to 8). In the third experiment, a limited number of replicate cultures was also prepared from virus-treated cells of the second experiment to see the effect of a double infection with polyoma. As noted earlier, the virus used in this experiment was recovered from infected cultures of the second experiment. No conspicuous differences in cytopathogenicity or rate of cell growth could be detected between the cultures receiving a double treatment and those that received a single treatment with the virus.

Independent cell lines of virus-treated and untreated cells of salivary clone 3435 were originated to study the effect of virus infection on 1) cell growth *in vitro* and 2) the capacity of the cells, when returned to mice, to grow as tumors. In each of 3 experiments, the virus-treated cultures grew more slowly than the untreated controls. Cultures of untreated control cells had to be transplanted about twice as often. Assays of cells at 0, 5, and 19 weeks after treatment are summarized in text-figure 2. The reduced rate of population increase in the virus-treated cultures appeared to result from a persistent, rather diffuse necrosis in the cell population.



TEXT-FIGURE 2.—Population growth in virus-treated and untreated cultures assayed at 0, 5, and 19 weeks after virus treatment. Average number of cell nuclei per culture is plotted against time in weeks. Averages are based on 3 cultures in each group.

Production of Tumors From Polyoma-Treated Cells

Both virus-treated and untreated cells of clone 3435 were implanted into mice to determine the effect of polyoma infection on capacity of the cells to grow as tumors. The results of these injections are summarized in table 1. In three experiments a total of 10 cell suspensions was prepared from 10 different groups of cultures. Of 15 mice inoculated with virus-treated cells, 10 developed tumors at the inoculum site. Inoculums ranged in size from 1.7 to 5.5 $\times$ 10^6 cells per mouse. Of 14 mice inoculated with comparable numbers or larger inoculums of untreated control cells, none developed a tumor. Inoculums ranged in size from 2.5 to 12.5 $\times$ 10^6 cells per mouse. However, in 2 mice tiny implants (about 2 mm in diameter) of fusiform cells located around the sciatic nerve or in extremely heavy collagen in the fascia were found on autopsy, 3 and 4 months after implantation of cells.

To be certain that the tumors originated from the implanted cells and not from the virus-infected host, we had implanted the culture cells into

TABLE 1.—Comparison of virus-treated and untreated culture cells (C3H) with respect to their growth as tumors in irradiated mice (C3H × C)F_1 or C3H*

	Virus-treated cells					Untreated cells	
Experiment No.	Weeks in culture before treatment	Weeks after treatment	Average inoculum $\times 10^6$ cells per mouse	Number of mice with tumors / Number of mice inoculated	Individual tumor latent periods (days)	Average inoculum $\times 10^6$ cells per mouse	Number of mice with tumors / Number of mice inoculated
1	40	13	2.3	2/2	25, 30	3.2	0/2
2	52	5	1.7, 3.4†	1/4	116	2.5, 5.0†	0/4
			2.1, 4.2†	3/4	39, 52, 52	4.5	0/5
3	57	6	2.8‡	2/2	31, 45	5.9	0/2
			5.5	2/3	53, 53	12.5	0/1
Total				10/15			0/14

*All mice used were hybrids with the exception of 2 mice in each group, a total of 8 in experiment 2. These were strain C3H mice, and 2 developed tumors when inoculated with 2.1×10^6 virus-treated cells.

† In experiment 2, 5 mice inoculated with virus-treated cells and 2 inoculated with untreated cells received the inoculum in 2 ml culture fluid instead of 1 ml. Three of these mice developed tumors.

‡Cells from experiment 1 treated a second time with virus in experiment 3.

(C3H × C)F_1 hybrid hosts. Tumors originating from host tissues would in general be transplantable to the hybrid host but not to C3H or C mice, whereas tumors originating from implanted C3H cells would be transplantable to C3H and hybrid hosts, but not to C mice (*34*). When 3 of these tumors were transplanted, all grew progressively in C3H and hybrid hosts, but failed to grow in C mice (table 2), which indicated that the tumors arose from the implanted cells and not from host tissues.

TABLE 2.—Transplantability of tumors produced by implantation of virus-treated cells

Experiment No.	Number of tumors transplanted	Strain of mouse developing tumor from injected culture	Transplantability expressed as: Number of mice with tumors / Number of mice inoculated		
			C3H	(C3H × C)F_1	BALB/c
1	1	(C3H × C)F_1	3/3	3/3	—
			2/2*	—	0/2*
2	2	(C3H × C)F_1	2/2	2/2	0/2
	3		2/2	—	0/2
	4	C3H	2/2	—	0/2
3	5	(C3H × C)F_1	2/2	—	0/2

*Third generation *in vivo* after 2 passages in (C3H × C)F_1 mice.

The tumors were exceedingly hard in consistency and grew progressively, certain ones attaining a diameter as large as 3 cm in 1 or 2 months.

Production of Tumors From Untreated Cells

At the time of these studies, cells of strain 2881 and 2661 from mouse embryonic tissue and adult connective tissue, respectively, had undergone malignant alteration in culture in the absence of any known carcinogenic agent. Results of injecting these cells into mice are summarized in table 3.

TABLE 3.—Tumor-producing capacity of cells after different periods of culture *in vitro*

Cell strain or clone	Generations in culture	Weeks in culture	Inoculum range $\times 10^6$ cells per mouse	Number of mice with tumors / Number of mice inoculated	Range of tumor latent periods (days)	Number of tumors subinoculated	Number of mice with tumors / Number of mice inoculated	Diagnosis of tumors
2881	0	0	—	32/57	12–19	6	1/12	Embryoma
	1, 3	4, 17	3–9*	0/2	—	—	—	—
	16, 20	54	3*	2/3	49–73	2	2/4	Sarcoma
2661	2, 3	36	0. 4*†	0/10	—	—	—	—
	18, 22	82	3*†	2/2	56	1	2/2	Sarcoma
	23–25	85, 91	3–13	4/4	10–76	1	4/4	Sarcoma
3435	18–22	53–62	3–13	0/14	—	—	—	—
	26, 27	68, 71	2–4	31/31‡	—	3	4/6	Sarcoma

*Estimated inoculum size based on one T-60 culture containing 3×10^6 cells.

†Mice less than 24 hours of age. The first group was of strain C3H and the second groups were (C3H × AKR) F_1 hybrids.

‡Not readily palpable tumors, but small growths resulting in paralysis and distortion of the left leg.

With both cell strains, a limited number of early inoculations made during the first 36 weeks of culture failed to produce tumors. Minced embryonic tissue such as that used to initiate cell strain 2881 was also inoculated directly into mice before any period of culture *in vitro*. Numerous embryomas composed of bone, cartilage, melanocytes, epithelial cysts, and other differentiated tissues developed from the implants. One of these tumors grew to small size on subinoculation. However, none of the sections showed any evidence of neoplasia.

Later injections of cell strains 2881 and 2661, made after 54 to 82 weeks of culture, regularly produced transplantable sarcomas. These sarcomas were exceedingly hard in consistency.

In view of these results, untreated cells of clone 3435 were also inoculated into mice after 68 and 71 weeks of culture, 6 to 18 weeks after the first 5 sets of injections. Thirty-one mice were inoculated. The inoculum

size was in the range of that used earlier (table 3). About a month after inoculation, all the mice showed deformity of the leg on the side receiving the injection. On autopsy, small, hard growths around the sciatic nerve or in the hip-joint area were observed.

To rule out the possibility of polyoma contamination of the culture cells or the mice, 6 of the mice bearing these growths were tested for polyoma infection by the hemagglutination-inhibition technique of Rowe, Hartley, Estes, and Huebner [5] (*45*). Mice were tested 1½ and 2 months after cell inoculation. All tests were negative, showing no evidence of polyoma infection.

Pathological Observations

On histologic examination, a notable feature of the tumors resulting from the implantation into the mouse of cells from the different culture lines was the large amount of collagen developed. Histologically, all the tumors were composed of fusiform cells resembling fibroblasts, and the cells were arranged in interlacing, thick strands. The amount of collagen varied in different areas of the tumors, and it was difficult to make a comparative estimate of the amounts. In general, collagen development appeared to be least in the tumors that arose after injection of cells of strain 2661 (derived from subcutaneous tissue), and it was relatively less abundant in tumors from injections of 2881 cells (derived from embryonic tissue) than in tumors from cells of clone 3435 (derived from salivary-gland tissue). When tumors from the polyoma virus-treated clone were compared with tumors derived from untreated 3435 tissue-culture cells, the most extreme degrees of collagen formation were found in the latter. Since these tumors represent the most extreme alteration, their effects upon the host and their morphology will be given.

Cells from the tissue cultures of untreated clone 3435 were injected into the muscles of the left thigh. In our previous experiences with most cell cultures, a palpable tumor mass developed at the site of transfer. With the present injections, however, a different result ensued. The first abnormality detected was a dragging of the foot on the side bearing the transplant. At a later period, the leg was misshaped and twisted and, in the most severely affected mice, the foot projected upward from the leg (figs. 9 and 10). The leg on the opposite side was occasionally affected, and normal motion at the hip was lost. The foot often did not respond to stimuli, and no independent motion of the foot could be elicited.

An explanation for this distortion and paralysis was revealed when histologic sections were studied. The neoplasms arising at the site of transfer developed a large amount of collagen and showed an extreme capacity for infiltration between muscle bundles and around nerve trunks. Muscles were replaced by a dense, collagenous tissue. Contraction occurred and extreme distortion of the leg resulted (fig. 11). Instead of producing a greater bulk of tissue, these tumors appeared to occupy a

[5] The authors express their appreciation to Dr. Mary Fink and to Mr. Malcolm B. Melroy of the Biology Laboratory of this Institute, who carried out these tests.

smaller space than the normal tissues they replaced, and to contract like scar tissue.

A nerve trunk in the muscles of the calf and peripheral to the tumor showed severe degenerative changes (fig. 12). A still intact nerve trunk in the thigh was surrounded by tumor cells and collagen which infiltrated between muscle bundles (fig. 13). Multinucleate muscle cells were often seen (fig. 14), an indication of attempted repair after injury (*46*). The femur from 1 mouse showed invasion and replacement of the bone marrow by fusiform tumor cells, but in this location formation of collagen was relatively sparse (fig. 15). A feature of the neoplasms was a variation in the amount of collagen formed in different areas. In the more peripheral areas, the neoplasm often showed a nodular growth and was composed of fusiform, fibroblastic cells with relatively little collagen (figs. 16 and 17). On transfer, the resulting neoplasms were composed entirely of this type of fibroblastic cells with very little collagen. They grew as firm, rounded nodules at the site of transfer and caused little or no paralysis or distortion of the leg.

DISCUSSION

This study explored the possibility of inducing a rapid and reproducible neoplastic conversion in a clone of cells during the period *in vitro* before "spontaneous" conversions occur. The results were encouraging and indicate the feasibility of this approach. Clearly, virus infection produced a change in the clone. Whether this change was produced by selection of more malignant, transplantable cells from the whole population, or whether the individual cells were altered by the virus must await further study. However, in either case, two populations of cells differing in neoplastic properties were obtained from replicate cultures in repeated experiments.

Dawe and Law (*47*) have reported morphologic changes in salivary-gland tissue of the newborn mouse exposed to polyoma virus and maintained as organ cultures *in vitro*. Proliferative as well as cytocidal effects were observed side by side in the same cultures as early as 14 days after virus infection, and the salivary-gland epithelium developed characteristics similar to those of salivary-tumor cells *in vivo*. Attempts to transplant the cultures to isologous hosts did not result in progressively growing tumors at the site of transplantation. However, in a continuation of this earlier work, one tumor has been produced (*48*).

Morphologic effects of polyoma-virus treatment other than the initial cytopathogenicity, some pleomorphism, and the persisting diffuse foci of necrosis have not been observed in this study. Indications of a stimulatory effect of the virus on cell growth in the first experiment were not clearly confirmed in the later ones. A cinematographic analysis of rates of mitosis in virus-treated and control cultures will be necessary to estab-

lish with certainty whether the virus stimulates cell mitosis *in vitro* as appears to occur in the treated cells implanted *in vivo*.

Vogt and Dulbecco (*49*) have recently described some effects of polyoma virus on short-term cultures of trypsinized whole embryos of Swiss mice and Golden hamsters. The hamster cultures, in contrast to those of the mouse, showed no obvious sign of cell degeneration, yielded a smaller average production of virus per cell, and showed a variable proliferative response with the development in some cultures of whorls of heavy cell strands. When cells ($1–4 \times 10^6$) of 6 independently transformed hamster-embryo cultures were inoculated under the skin of 18- to 20-day-old hamsters, all 6 gave rise to progressively growing fusiform-cell tumors, 5 palpable as early as 6 days. A large number of hamsters of the same age inoculated with comparable numbers of uninfected cells or with 5×10^7 to 10^8 pock-forming units of polyoma failed to develop tumors. Tumor tissues transplanted to culture showed in the outgrowth the same type whorls observed in the original cultures, but in our experience such whorls are common in dense cultures of fibroblasts or sarcoma cells. These workers concluded that an oncogenic effect of the virus had been produced in culture, and their results seem to indicate such a change.

The hamster system may be preferable to the mouse system reported here, since cytopathogenic effects resulting in cell selection have not been observed in the hamster cultures. On the other hand, the local oncogenic response of hamster tissue to polyoma virus as reported by Stanton (*50*) makes it appear possible that, in assaying virus-infected cells in the hamster, the resulting tumors may arise from the host tissues and not from the implanted cells. Identification of the tumor cells is, therefore, important, and can be carried out by the use of mouse cells from an inbred strain as indicated in this paper.

In the present study, three strains of mouse cells, all of the same original genotype, have been compared under similar culture conditions with respect to their response to treatment with polyoma virus. In the long-term strain of embryonic cells, cytopathogenic effects were observed, as has been reported previously for short-term cultures (*51*), but some cells could always be recovered and maintained as long-term strains. Of the 2 adult cell types originating from subcutaneous connective tissue and from salivary-gland tissue, only the latter appeared susceptible to the necrotizing effect of the virus.

C3H mouse cells, some containing the mammary-tumor agent and some agent-free from several different tissues (connective tissue, skeletal muscle, liver parenchyma, and skin epidermis), have been found to undergo malignant conversion after prolonged culture *in vitro* (*14*, *16*, *17*, *52*). All these cells were grown in an undefined medium containing horse serum, and a saline extract of 9- to 10-day chick embryos. Under these conditions, the cells may have been exposed to unknown carcinogens, viruses, or differentiating agents, particularly in the chick-embryo extract. Bang (*26*) has reviewed evidence for the frequent occurrence of virus particles in normal chick embryos, and Gaillard (*53*, *54*) has shown that

extracts from different age embryos can affect the differentiation of cells in culture. The present study is significant[6] in showing that agent-free or agent-containing cells cultured in the absence of chick-embryo extract, in a serum-supplemented, chemically defined medium, also undergo a malignant change.

No entirely satisfactory explanation can be proposed for the excessive amounts of collagen found in the tumors developing after the injection of tissue-culture cells from the four different sources: 1) embryonic tissue, 2) subcutaneous tissue, 3) a clone from salivary-gland tissue treated with the polyoma virus, and 4) untreated cells from the same clone. As indicated, fibrosarcomas have been produced repeatedly in mice after the injection of cells grown in tissue culture. These usually have been bulky tumors composed of fusiform cells with sparse collagen formation. In the present groups of tumors, particularly in the tumors developing after the injection of cells from the untreated salivary-gland clone, the formation of collagen equaled or exceeded the amount usually formed in a healing wound, and contractures such as result from scar tissue developed.

Whether the collagen was developed from the host tissues or by the neoplastic cells cannot be definitely answered. The formation of collagen is a function of the fibroblast, and neoplastic cells may continue the function of the normal cell, as illustrated in keratin formation in epidermoid cancer or hormone production in various endocrine tumors. On the other hand, some neoplasms may induce a fibrous tissue reaction in the host as in some cancers of the breast or stomach in man. The lack of collagen formation when the tumor invaded the bone marrow suggests that it was the host's tissue from which the collagen was derived.

One significant feature of the present tissue-culture study is the employment of a serum-supplemented, chemically defined culture medium. Cells grown under these conditions may produce tumors that grow more slowly and appear more highly differentiated and less malignant than tumors derived from cells grown in a medium containing embryo extract. The possibility that the collagen formation resulted from the culture fluid alone can be eliminated in view of the many injections of cells in this type fluid into similarly irradiated adult mice that failed to show any such collagen development.

Although the tumors derived from the salivary-gland clone resembled fibroblastic tumors, the possibility should not be overlooked that the fusiform cells of the tumor were modified salivary-gland epithelial cells. Two types of cell morphology were seen in the tissue-culture clone (figs. 4 and 5), and the tumors arising on transfer to the mouse may have been composed of modified epithelial cells.

The second generation transplant of the tumors no longer showed the

[6] At the Tenth International Congress on Cell Biology in Paris, 1960, Dr. V. J. Evans and Mr. G. Parker of this Section reported malignant transformations in other strains of C3H mouse-embryo cells cultured in serum supplemented, chemically defined medium. The detailed study, which also delimits the time of transformation, is now being prepared for publication by these workers.

collagen formation and contractures observed after the transfer directly from tissue culture. This loss of distinguishing characteristics with continued transplantation has been observed in many tumor lines.

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PLATE 36

FIGURE 1.—Area of clone culture photographed 24 days after isolation of single cell. *Note* sheetlike outgrowth from cells in capillary tube. × 200

FIGURES 2 AND 3.—Representative areas of untreated and virus-treated culture of clone 3435 (figs. 2 and 3, respectively) photographed 8 days after addition of virus. *Note* beginning of necrosis in treated culture. × 200

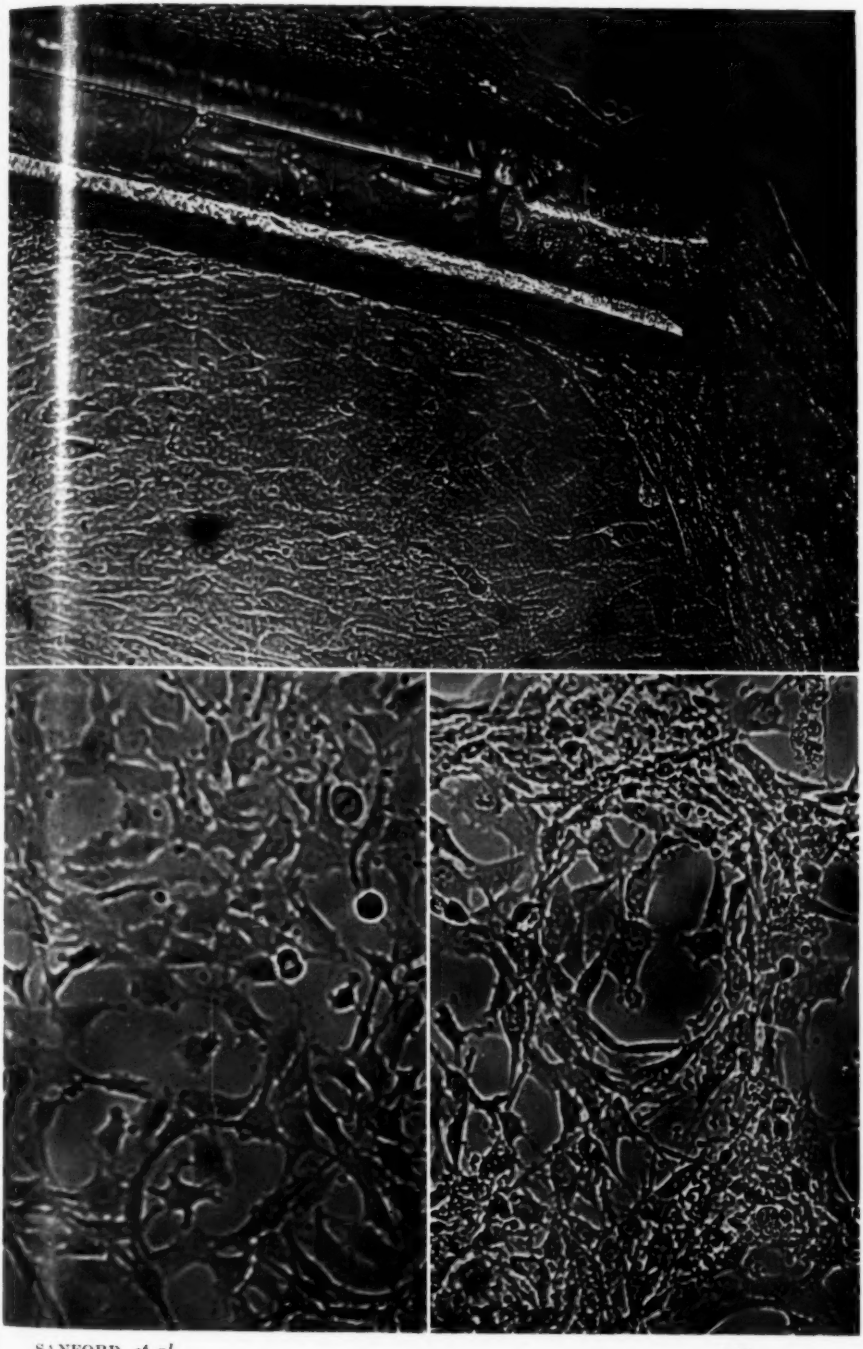

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PLATE 37

FIGURE 4.—Culture of clone 3435 showing epithelium-like morphology characteristic of both virus-treated and untreated cells. This culture was photographed 9 months after single-cell isolation. × 200

FIGURE 5.—Culture of clone 3435 showing fibroblast-like morphology characterizing active growth phase of both virus-treated and untreated cultures. This culture was photographed 6 months after single-cell isolation and 8 days after addition of virus. × 200

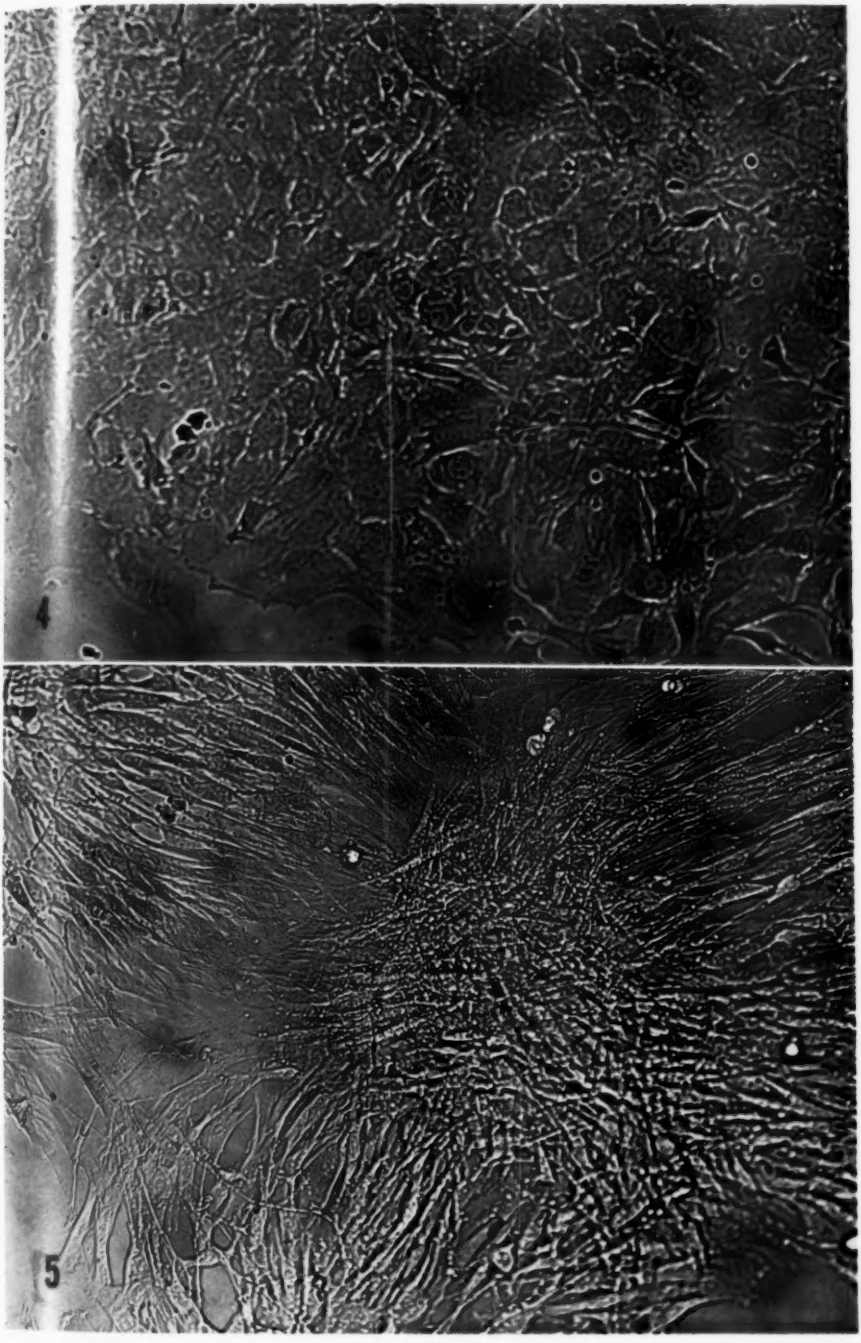

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PLATE 38

Virus-treated and untreated cultures of clone 3435, fixed in Zenker-formol and stained with iron hematoxylin. × 235

FIGURE 6.—Untreated cells.

FIGURE 7.—Virus-treated cells. Area of culture showing cell disintegration and pleomorphism, 5 days after addition of virus.

FIGURE 8.—Virus-treated cells. Area of culture showing less conspicuous effects of virus treatment, though many necrotic cells had been removed. Culture was fixed 12 days after addition of virus.

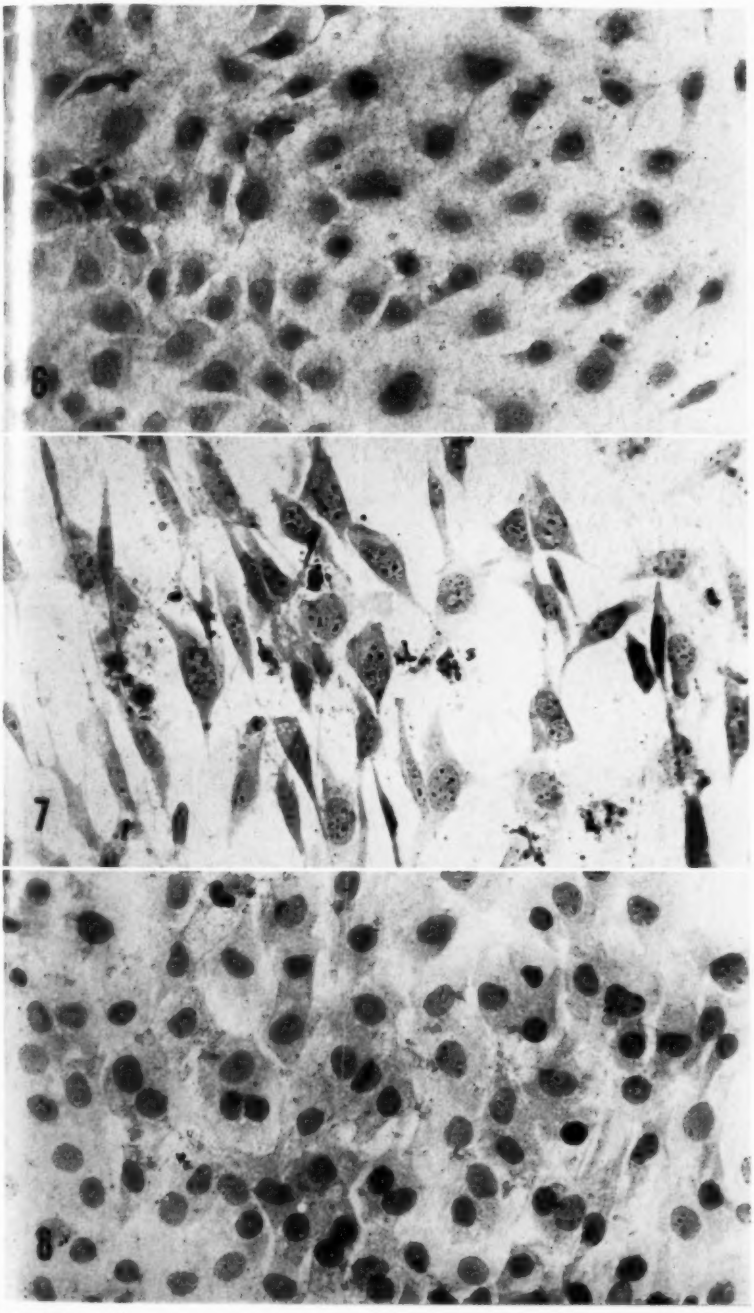
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PLATE 39

FIGURE 9.—Four mice showing dragging or distortion of left hindleg (*arrow*) photographed 3 months after inoculation of clone 3435 cells. *Observe* bilateral involvement in mouse to extreme *left* and marked distortion of hindleg in *second* mouse from *left*.

FIGURE 10.—Mouse with leg so misshaped and twisted that foot projects upward from leg.

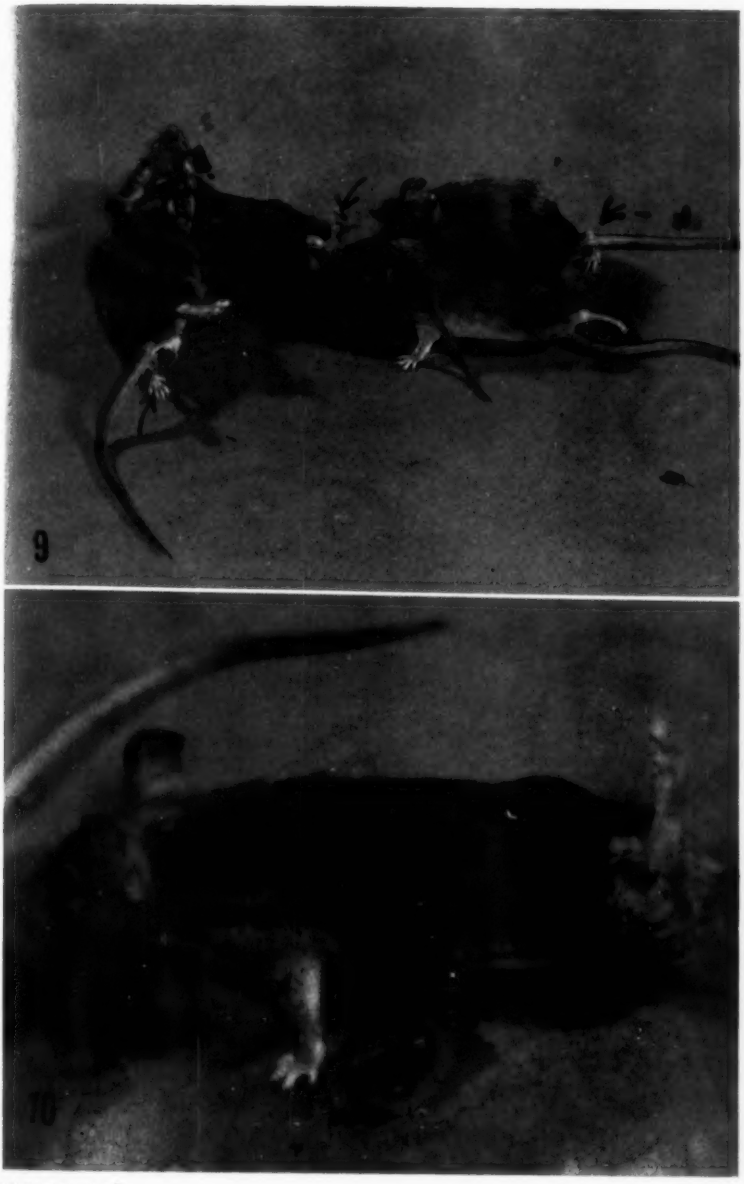
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PLATE 40

FIGURE 11.—Section through muscles of thigh and calf in region of knee joint. Cells from tissue culture of salivary gland, not exposed to polyoma virus, were injected into muscles of thigh. Large amount of collagen has formed and strands of tumor tissue have infiltrated and destroyed muscle, producing contracture at the joint. van Gieson's stain. × 13

FIGURE 12.—Nerve trunk in the calf muscles, from same section as above. *Note* degenerative changes secondary to compression of proximal portion of nerve by tumor. van Gieson's stain. × 420

FIGURE 13.—Nerve trunk in thigh, surrounded by fusiform tumor cells and dense strands of collagen. Nerve fibers at this level show no degeneration. van Gieson's stain. × 260

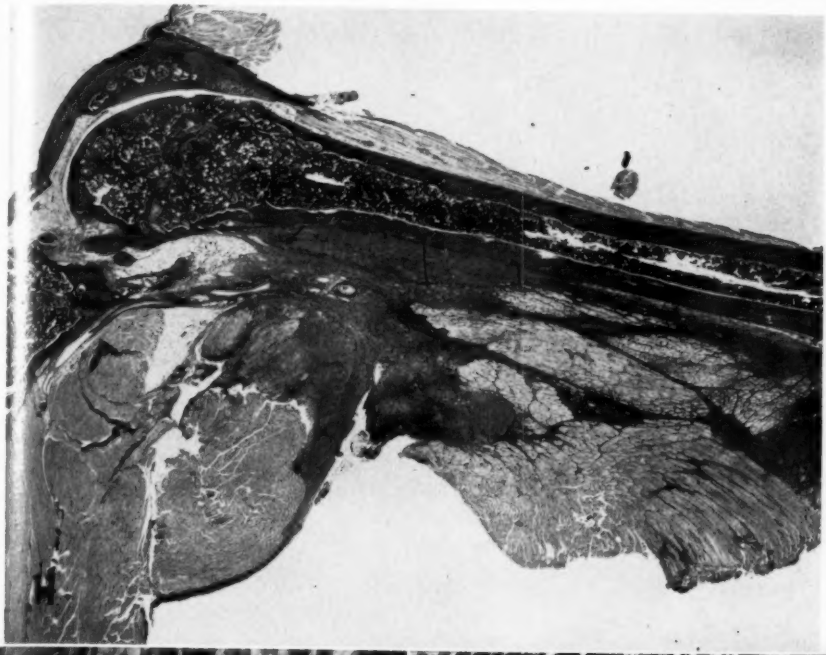


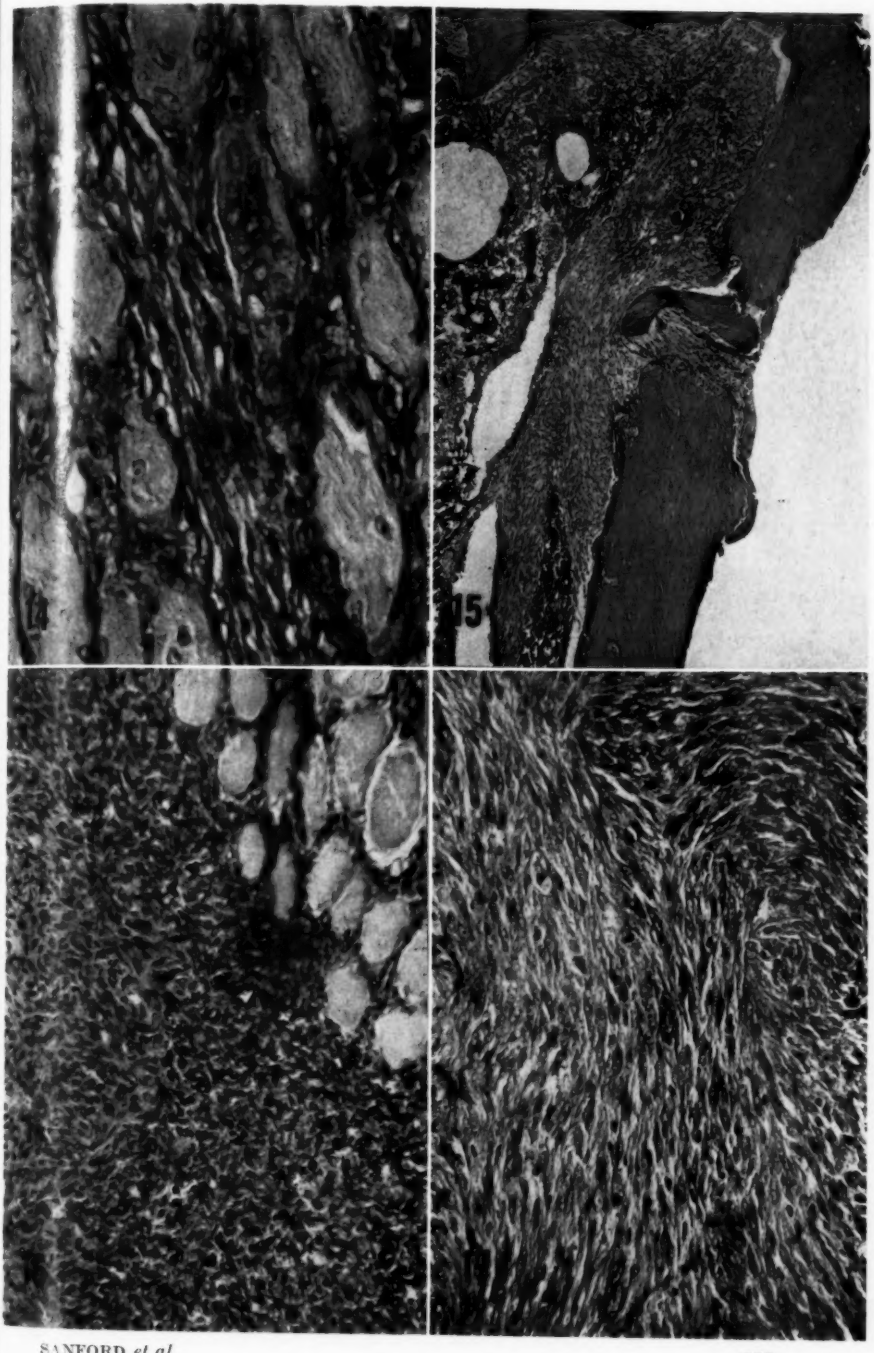
PLATE 41

FIGURE 14.—Muscle fibers separated by fusiform tumor cells and dense strands of collagen. Some muscle fibers have been destroyed and others show multiple nuclei. van Gieson's stain. × 420

FIGURE 15.—Tumor infiltration in marrow cavity replacing hematopoietic tissue on *right*. Relatively little collagen has been formed. van Gieson's stain. × 50

FIGURE 16.—Infiltration of muscle fibers by collagenous tumor tissue at *upper right*. Amount of collagen decreases and tumor appears more cellular on *left*. van Gieson's stain. × 210

FIGURE 17.—A more cellular area in a tumor, with scant collagen formation. van Gieson's stain. × 210



Virus of Avian Erythroblastosis. VII. Ultrastructure of Erythroblasts From the Chicken and From Tissue Culture [1, 2]

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SUMMARY

Studies have been made on culture *in vitro* of the erythroblasts occurring in the circulating blood of chickens with erythroblastosis. In 23 experiments during the past 3 years, growth of the cells was observed for more than 80 days in 3 of the studies. Cell proliferation has been dependent on a culture medium consisting of 50 percent normal chicken serum in medium 199 which contained, in the more recent experiments, vitamins in the concentration found in Eagle's medium. Glucose was added to medium 199 in a concentration of 0.5 percent. The average generation time measured in 1 experiment was 9.9 days. Concurrent with maintenance and growth of the cells in culture, erythroblastosis virus was liberated continually for periods up to 125 days, as demonstrated by electron micrography of thin sections. The observation of typical virus particles in the culture fluids was corroborated by the induction of the characteristic erythroblastosis in susceptible chickens. An investigation was made also of the ultrastructure of erythroblasts and other cells in the diseased bird and of the erythroblasts after various periods in tissue culture. Numerous cells of the character of macrophages, and reticular and endothelial cells were seen in the bone marrow and spleen which contained large numbers of virus particles in vacuole-like structures. Virus was seldom observed in the erythroblasts in the tissues or in the circulating blood. Most of the virus in the erythroblasts under these conditions was collected in vacuoles, but an occasional virus "bud" occurred in the cell membranes. Erythroblasts in the circulating blood exhibited increased vacuolization. The culture of erythroblasts resulted in little, if any, increase in virus associated with the cell. Some cells contained collections of virus in vacuoles, which had the appearance of originating by the process of phagocytosis of the agent seen in considerable amounts outside the cell. The probability of the occurrence of phagocytosis was supported by the demonstration that the erythroblasts in the cultures ingested large numbers of India ink particles, among which virus particles were seen in the vacuoles. Buds, which had the appearance of formation of virus particles, were seen frequently in the cell membrane. The particles in the process of formation contained no nucleoids. The findings were interpreted to indicate synthesis of the virus in immature form in the cell membrane and maturation of the agent with formation of the nucleoid outside the cell. No evidence was found to indicate the synthesis of

[1] Received for publication August 8, 1960.

[2] This work was aided by research grants to Duke University from the National Cancer Institute, National Institutes of Health, Public Health Service; from the American Cancer Society, Inc.; and by the Dorothy Beard Research Fund.

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virus in mitochondria or any other cellular structure. The results were discussed in relation to findings with myeloblasts in which the specific virus is synthesized in walled bodies inside the cell by processes not involving the cell membrane.—J. Nat. Cancer Inst. 26: 359–388, 1961.

MYELOBLASTOSIS (*1*) and erythroblastosis (*2*) are avian leukemias transmissible by separate and distinct, though immunologically closely related, viral agents (*3*, *4*). Host response to the virus of myeloblastosis results specifically in a rapid overgrowth of primitive cells of the characteristics of myeloblasts (*1*) which exhibit to a high degree the attributes of malignancy. In contrast, host response to the erythroblastosis virus is marked by the occurrence in the circulating blood of primitive cells (*2*) which are morphologically indistinguishable from erythroblasts observed in the normal chicken. Despite numerous studies of these two viruses involving inoculation of many thousands of chickens, routine examination of blood smears taken at frequent intervals on every bird has revealed no evidence that the myeloblastic cell system could respond to the erythroblastosis virus or vice versa.

Because of the evident kinship between these leukemia viruses which affect totally different cellular elements, an opportunity is afforded for the investigation of virus-host cell relationships in the infectious processes involving the respective agents. Much has already been accomplished in this respect in the study of myeloblastosis. The myeloblasts from diseased birds proliferate rapidly in tissue culture (*5–8*) and under these conditions the virus is synthesized and liberated constantly into the culture fluid. Electron microscopic examination of the cells (*6*, *9*, *10*) has provided the basis for identification of specific organized bodies responsible for virus synthesis and for correlative cytochemical analyses (*10–12*) of the constitution and activity of the bodies by techniques of light microscopy.

It is desirable to bring the knowledge of analogous phenomena of erythroblastosis into parallel with that of myeloblastosis. An approach to this problem has been made in the present work, principally by electron microscopic study in thin sections of erythroblasts from the circulating blood of diseased chickens and of erythroblasts after various periods in tissue culture. The results have confirmed and extended the findings of other investigators (*13–17*) working by similar methods with erythroblastosis in the diseased bird. The manifestations of host-cell response to this virus differ profoundly in nearly every respect from those of myeloblasts to the myeloblastosis virus. Although the virus-diseased erythroblasts behave as malignant cells, growth in tissue culture is more difficult to establish, and the cells do not thrive as well *in vitro* as do the myeloblasts. As the present work and that of others (*13–17*) have shown, there is relatively meager evidence of the sites of proliferation of the virus, and exploratory cytochemical studies (*11*) have not revealed specificity of cell organelle involvement in the processes of virus synthesis. It is the pur-

pose of this report to describe the results thus far obtained and to compare them with the findings of similar studies with myeloblastosis.

MATERIALS AND METHODS

Erythroblastosis virus used to produce the disease under study was the Engelbreth-Holm strain "R" obtained (*18*) in 1955 from Dr. Astrid Fagraeus. The agent has been passed many times in this laboratory, always by means of previous-passage blood plasma filtered through Selas #02 candles. Cells or tissues for immediate study and for tissue culture were obtained from inbred White Leghorn chickens of line 15, Regional Poultry Research Laboratory, East Lansing, Michigan (*19*), inoculated intravenously at about 4 months of age with 0.1 ml volumes of pools of previous-passage plasma in dilutions from 10^{-1} to 10^{-3}. Host response to the virus has been uniform and has resulted in the occurrence of typical erythroblastic leukemia as determined from blood smears made in the manner and at intervals as already described (*18*).

Tissue cultures of erythroblasts were prepared and maintained by the principles of handling used for the culture of myeloblasts (*8*). Chick donors of the cells were selected on the basis of the number of erythroblasts in the circulating blood as judged from those seen in blood smears. Because of the relatively low concentration of cells in the blood (not more than $5 \times 10^5/mm^3$) it was necessary to use several donor birds for each experiment. Blood was drawn by heart puncture into tubes containing heparin (*8*) and centrifuged for 10 minutes at $150 \times g$. The plasma was pipetted off, and the layer of erythroblasts, containing large numbers of red blood cells, was removed. The cells from the donors were pooled and, in most instances, were resuspended in a portion of the plasma from the same birds. Centrifugation of this suspension for 10 minutes at $150 \times g$ resulted in a further separation of erythroblasts from the red blood cells. The layer of erythroblasts, still containing about 20 percent red blood cells, was suspended in culture medium. We prepared cultures by adding about 2×10^6 erythroblasts to 5.0 ml of medium in 50 ml Erlenmeyer flasks which were closed with rubber stoppers and incubated at 37° C on an oscillating shaker. Culture fluids were changed at intervals of 2 to 3 days by the techniques employed for myeloblast cultures. In only a few series of cultures was cell proliferation sufficient to permit culture division. In experiment 10458, division was made routinely when the packed volume of cells reached levels of about 0.08 ml, approximately 1.5×10^6 erythroblasts per culture. New cultures were established with about 0.04 ml packed cells per 5.0 ml culture fluid.

Thin sections were made on cells from the bone marrow of normal chickens, bone marrow and erythroblasts from the circulating blood of birds diseased with the erythroblastosis virus, and erythroblasts from the

circulating blood after various intervals in tissue culture. Examinations were made of erythroblasts from 7 cultures which were derived over the course of 2 years from chickens diseased with virus from 3 different sources. Of these, 2 were large pools of plasma, "P" and "R", collected from previous-passage, diseased hosts and stored under cracked CO_2 ice. In experiment 260 the cells for culture were obtained from birds diseased typically with virus liberated into the culture medium by erythroblasts established in culture (expt. 10458) 22 days earlier.

Preparation of the various materials for electron microscopic study was effected as described (*10*) for similar examinations of myeloblasts. In most instances the sections were stained with lead.

Bioassay for biologic estimate of amount of virus, in terms of particle number, was made by the procedures devised (*18*) for titration of the erythroblastosis virus. The standards for comparison were pools of plasma, stored under cracked CO_2 ice, from previous-passage birds. Determinations of particle number in the standards were made by sedimentation of the virus on agar and direct counting [3] in the electron microscope (*20*). For the tests in chickens, the standard plasma and fluid were inoculated in 0.1 ml volumes of appropriate dilutions into the wing vein of chickens about 3 weeks of age. Each dose group of standard and test material consisted of 30 chickens. Blood smears were made in the usual way (*18*) to confirm the occurrence of the disease, and the time of death of the bird was the criterion of length of the latent period. With the potency ratio of the test fluid and standard, the number of particles in the former was calculated.

RESULTS

During the past 3 years, 23 separate experiments have been made with various media and under different culture conditions in the effort to establish erythroblasts in tissue culture. In experiments 5257, 10458, and 2560, the cells have been maintained for more than 80 days, and in experiments 10458 and 2560 cell growth was sufficiently great to permit repeated division of the cultures. The cells of experiment 2560 have continued to grow for 91 days. Despite numerous trials, it has not yet been possible to obtain persistent growth of erythroblasts comparable with that of the myeloblasts (*8*). The results observed thus far have sufficed, nevertheless, to establish certain principles of behavior of the erythroblasts as a basis for further experimentation on tissue culture of these cells and as the foundation for interpretation of the ultrastructural studies to be described.

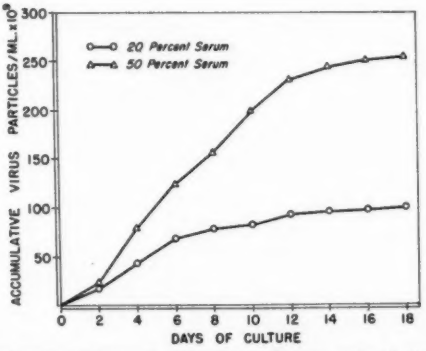
The initial experiments were made with culture medium now recognized (*8*) to be grossly deficient in nutrient and growth factors. In the beginning the medium consisted of normal chicken serum in different concentrations in Gey's salt solution, under which conditions the erythroblasts could be

[3] The authors acknowledge indebtedness to Dr. D. G. Sharp for counting the virus particles.

maintained for various periods but without increase in the total number of living cells.

We observed, however, that despite this state of the cultures, the erythroblastosis virus was released continuously into the culture fluid. We demonstrated this by electron micrographic examination of the fluid and by the induction of characteristic erythroblastosis in chickens inoculated with the culture fluid. By means of bioassay, it was noted, as in the work with myeloblastosis, that the rate of virus release was greater in *flask cultures* incubated on an *oscillating shaker* than in stationary flasks of the same sort or in stationary T-60 flasks (*5*) in which the fluid layer was very thin. The concentration of chicken serum exerted a great influence, as shown by the results of the following experiment.

A series of 6 cultures was prepared from the cells of 2 chickens 70 days of age. In the culture medium of 1 group of 3 cultures, the concentration of chicken serum was 20 percent and in the other 3 cultures, 50 percent. The respective culture fluids were renewed at the points indicated in text-figure 1, and the amount of virus in the fluid removed was measured by bioassay. The results shown in text-figure 1 illustrate the considerably greater output of virus in the presence of the higher concentration of serum. Other studies revealed that virus release by cells in 5 percent serum was small and ceased after about 8 days. Cells cultured in Gey's salt solution without serum released only insignificant amounts of virus throughout the culture period.



TEXT-FIGURE 1.—Influence of serum concentration on rate of liberation of erythroblastosis virus in tissue cultures of erythroblasts.

The influence of glucose concentration on the rate of virus release was the same in principle as that affecting liberation of myeloblastosis virus (*5*). A concentration of 0.5 percent of the carbohydrate in the serum supplement was employed for subsequent routine culture.

Virus Release in Prolonged Cultures

That the specific etiologic agent of erythroblastosis was released in considerable amounts by erythroblasts, even in cultures showing little if any

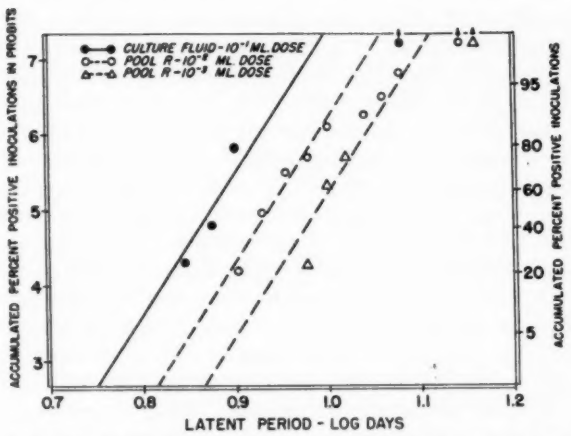
increase in the total number of cells, has been well established. This was shown by the results obtained in the following experiment.

Cultures were prepared in a concentration of 3.28×10^7 cells per ml of culture fluid consisting of 20 percent chicken serum in Gey's solution with added glucose in 0.5 percent concentration. The culture fluids were changed at intervals of 2 to 4 days for 86 days, each fluid was bioassayed as already described, and the number of virus particles in the respective fluids was calculated.

The results of the bioassays, which included a total of 97 dose groups and 2,903 chickens for the standards and test fluids, are given in table 1. The output of virus was high during the early stage of the cultures but diminished finally to approximately 10^7 particles per ml of fluid in each 4-day interval of culture. The content of cells remained at levels of about 9×10^6 per ml. No estimates can be made relative to the accuracy of particle measurement by this procedure. It is probable, however, that, because of presumable loss of infectivity of the agent during the culture intervals of 48 to 96 hours at 37° C, the values were low. In consequence it can be estimated that the virus was being liberated at average rates of at least 20 to 70 particles/viable cell/hour during the initial periods of the experiment and approximately 1 particle/hour during the latter stages. Of more importance was the clearly defined evidence that the disease induced by the agent was typical erythroblastosis and that host response manifested by the characteristics of the dose-response curves (text-fig. 2) was likewise typical.

TABLE 1.—Virus released by erythroblasts during 86 days in culture in experiment 5257.

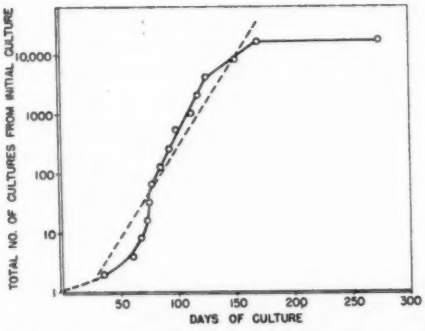
Days	Infective virus particles/ml of culture fluid ($\times 10^9$)
0–2	34. 7
2–4	114. 5
4–6	34. 7
6–8	160. 7
8–12	27. 1
12–16	5. 1
16–20	4. 9
20–24	0. 9
24–28	0. 6
28–32	0. 2
32–36	0. 4
36–40	0. 1
40–44	0. 01
44–48	0. 01
48–52	0. 006
52–56	0. 01
56–60	0. 004
60–64	0. 03
64–68	0. 03
68–72	0. 03
72–76	0. 003
76–80	0. 03
80–86	0. 02


CULTURE FLUID - 10^{-1} ML DOSE
POOL R - 10^{-2} ML DOSE
POOL R - 10^{-3} ML DOSE
ACCUMULATED PERCENT POSITIVE INOCULATIONS IN PROBITS
ACCUMULATED PERCENT POSITIVE INOCULATIONS
LATENT PERIOD - LOG DAYS

TEXT-FIGURE 2.—Time frequency distributions of chick-host responses to 2 dilutions of the standard erythroblastosis virus plasma (pool R) and to undiluted tissue-culture fluid of experiment 10458 taken on the 22d day of culture.

Growth of Erythroblasts

Improvements in the culture medium have provided conditions necessary for considerable growth of the erythroblasts. The findings in experiment 10458, carried for 275 days, are shown in text-figure 3. This study was important because examinations of the ultrastructure of the cells were made at frequent intervals from the 35th to the 125th day of culture. The cultures were prepared with pooled erythroblasts from the circulating blood of 5 chickens diseased with the virus by inoculation with filtered plasma obtained in previous passage of the agent.



TEXT-FIGURE 3.—Growth of erythroblasts in tissue culture in experiment 10458. Division of the culture was made at time of doubling of the number of cells which occurred at intervals indicated by *circles*. Growth is charted in terms of number of cultures which would have resulted, had all been kept. *Broken line* was drawn by the method of least squares and indicates an average generation time of the cells of 9.9 days.

A volume of 0.7 ml of erythroblasts was used to prepare 10 cultures of 5.0 ml volume each (about 2×10^7 cells per ml of culture fluid). The medium at the beginning of the experiment was the same as that used with the myeloblasts at the time (Dec. 16, 1958) and consisted of 20 percent normal chicken serum in medium 199 (*21*) to which glucose was added in 0.5 percent concentration. At 35 days the concentration of chicken serum was increased to 50 percent, and at 206 days the medium was supplemented with vitamins to the same level of concentration as that in Eagle's medium (*22*). These changes were made on the basis of the observation of the influence of such media on the growth of myeloblasts.

During the initial period of culture, there was evidence of some cell multiplication, as indicated by the presence of mitotic figures, but the rate of cell death was approximately the same as that of proliferation, so that the concentration of viable cells, determined with the trypan blue technique (*5*), remained essentially unchanged. After the change to 50 percent concentration of chicken serum, however, there was such an increase in growth that division of the cultures could first be made on the 35th day. Division was made thereafter by the procedures employed with the myeloblast cultures (*5*), at the intervals indicated by the points of the curve of text-figure 3. The cultures were divided 14 times, but growth at the same rates did not continue after the 125th day, and the experiment was terminated on the 275th day. The discontinuous line drawn by the method of least squares through the experimental points indicated a mean generation time of 9.9 days. Specimens of cells for various studies, including those on ultrastructure, were taken at frequent intervals to the 125th day of culture.

Infectivity and specificity of virus in the culture fluid were demonstrated by bioassay in experiment 10458 only at the 22-day interval. From the results analyzed as described before, text-figure 2 shows that the characteristics of host response were essentially identical with those observed with this agent, as exemplified [*see* text-fig. 3 of (*18*)] by the results of earlier work. The potency of the culture fluid was the same as that which would have been expected with the undiluted standard of pooled plasma which contained 1.7×10^{10} virus particles per ml by electron micrographic count. By consideration of the degree of dilution effected by the changes in culture fluid, it is evident that the cells, though growing slowly in this period, were liberating virus at rates which resulted in concentrations of the agent in the culture fluid equivalent to those observed in the plasma from birds with erythroblastosis.

Ultrastructure

The pathologic aspects of erythroblastosis have been studied at the ultrastructural level by several investigators (*13–17*). Thus far, the investigations have been concerned entirely with material from bone marrow and spleen and with the primitive cells, erythroblasts, occurring in the circulating blood. Benedetti and Bernhard (*14*) confirmed the

earlier observation (*23*) of virus of typical structure in thin sections of material sedimented from the plasma of birds with the disease. Particles of identical appearance were seen in reticular cells and macrophages of the spleen and bone marrow. Frequently, larger numbers of these were packed in inclusion-like bodies regarded as derivatives of mitochondria. Other particles were in simple vacuoles thought to be the result of phagocytosis of virus demonstrable in the extracellular spaces. The vacuoles likewise contained fragments of red blood cells and other debris. In the erythroblasts themselves virus was infrequently demonstrable. As in the reticular cells and macrophages, virus occurred in simple vacuoles and was seen in structures interpreted (*14*) as altered mitochondria in which the agent was presumed to be in the process of synthesis. There was observed occasionally near or in the cell membrane another and quite different feature considered to be related to virus formation: vesicles of the approximate size of the virus just beneath the cell membrane, the occurrence of particles of high density within the membrane, and the protrusion of such structures into mounds extending above the membrane. The studies by Iwakata (*15*, *16*) were similar to the foregoing. This author, however, interpreted his results to indicate that the vesicles occurring near the cell membranes in the erythroblasts were precursors of virus which, included in castoff substances of the cell, were phagocytized by macrophages in which the final stages of virus synthesis were completed.

The results of the present work did not reveal any feature of erythroblastosis, as it occurs in the host, essentially different from that already described. This includes the observation that virus in identifiable form was seldom intimately associated with the erythroblasts in the tissues or those present in the circulating blood. The findings obtained with material from normal chickens and birds with erythroblastosis are illustrated in figures 1 to 3 (*see also* fig. 8).

Examinations were made first on bone marrow, spleen, and other tissues from normal chickens and chickens with erythroblastosis, with particular attention given to the erythroblasts. Figure 1 shows, for purposes of comparison, the appearance of an erythroblast from the bone marrow of a normal chicken at 4 weeks of age. The nuclei of such cells were relatively large and bounded by a double membrane in which nuclear pores (*24*) were visible in some sections. The chromatin material was finely divided and evenly distributed throughout the nucleus. Nucleoli of finely granular substance were demonstrable in a few cells. The cytoplasm was a comparatively thin layer of relatively homogeneous constitution, containing only sparsely scattered, specific, differentiated organelles represented by ergastoplasmic lamellae, mitochondria, lipochondria, and a poorly developed Golgi apparatus. The homogeneity of the cytoplasm was emphasized by the uniformity of distribution of the Palade granules which were not notably associated with a specifically formed lamellar system. The mitochondria were few and of oval or elongated shape. The cristae were sharply defined, and their arrangement in the organelle exhibited no characteristics peculiar to the erythroblasts. Some of the cells showed

simple vacuoles containing ferritin. The cell membrane was not exceptional in appearance and did not exhibit evidence of special activity such as might have been shown by the occurrence of pseudopodial formations or invaginations associated with unusual degrees of phagocytosis or pinocytosis.

We were surprised to learn that the erythroblasts in the marrow of chickens with advanced erythroblastosis showed so little structural sign of disease processes within the cell or of response to disease involving the cells. This is evident from the cell shown in figure 2 from a chicken 9 days after inoculation with the virus. In the appearance of the nucleus, the cytoplasm, mitochondria, and other cytoplasmic structures, we could detect no essential difference from the normal cell pictured in figure 1. One aspect of these cells, though, was particularly notable. Membrane activity was marked by numerous pseudopodial protrusions and membrane irregularities. In such cells, in general, there were rather large numbers of small vacuoles situated beneath the cell membrane which were undoubtedly associated with accentuated phagocytic and pinocytic activity. In sections of this sort, virus particles were frequently seen in the intercellular spaces.

The structure of the large number of erythroblasts in the circulating blood of the chickens with leukemia differed from those in the tissues. As already noted, the number of these cells containing virus was exceedingly small. In these cases the virus was usually enclosed in structures of the appearance of simple vacuoles with a single wall (fig. 3). Together with the virus, there were variable amounts of amorphous material without any evidence of organization. This structure strongly suggests that it was derived by phagocytosis of extracellular virus particles and debris either in the circulating blood or in the tissues before the cell reached the blood stream. This was supported by micrographs of erythroblasts from tissue culture (figs. 4, 5, and 6). The nuclei were like those of the tissue cells. The cytoplasm, however, exhibited numerous vesicles and vacuoles containing bodies of indeterminate character. The mitochondria were frequently swollen. Note especially in figure 3 the occurrence of the budlike process at the *lower left.*

Erythroblasts in Tissue Culture

In contrast with the relatively small evidence of deviation from the normal of the structure of erythroblasts in the diseased bird, extensive changes occurred in the cells transferred to tissue culture. Some of these are illustrated by the cells shown in figure 4 after tissue culture for 11 days. Clumping and margination of the chromatin (*24*) are evident in the nucleus, and the nucleolus of high contrast is clearly shown. In the cytoplasm there occur numerous large and small vacuoles. Occasionally these were one third to one half the volume of the cell. The centriole was found in a relatively vacuole-free region, and the Golgi apparatus showed poor differentiation. The mitochondria were typically swollen. While the

cristae were recognizable, the orderly arrangement was not evident. Pseudopodia and membrane invaginations were frequently demonstrable. Outside the cell, large numbers of virus particles were collected, mostly in a matrix of amorphous material presumably of precipitated protein.

In view of the observation of profound changes occurring in myeloblasts in tissue culture manifested by development of numerous intracellular bodies involved in virus synthesis, it was disappointing to observe no evidence of an analogous phenomenon in erythroblastosis. Instead, no more virus was seen in the cells in culture than in those in the chicken, except for a specific relationship described later. Most of the intracellular virus was located in vacuoles (fig. 5) like those in the circulating cell illustrated in figure 3. It was suspected that such structures and accumulations of virus were the result of phagocytosis. The pictures of figure 5 suggest probable stages in the course of ingestion of the virus particles and amorphous material demonstrated in figure 4. In figure 5A two large clumps of the material and of virus particles partly enclosed by cellular processes may be seen. Three masses of similar character lie in vacuoles within the cell pictured in figure 5B.

This apparently highly phagocytic behavior seemed greatly out of character for cells representing the precursors of erythrocytes. To test for this capacity directly, erythroblasts in a growing culture for 80 days were incubated for 2 days under routine conditions with a 1 percent suspension of washed India ink particles. In figure 6, the phenomenon demonstrated in figure 5 was duplicated almost precisely with the India ink particles. The extracellular particles observed in figure 6A have been partially engulfed by the cell, and in figure 6B the ink particles lie completely enclosed in a vacuole. In some cells, we saw an occasional virus particle in association with the carbon.

Evidence of an intimate association of the virus and the erythroblast, other than that illustrated in figures 4 and 5, was not often encountered in the tissue-culture material. Benedetti and Bernhard (*14*) have described an apparent relationship of particles and vesicle-like structures to the cell membrane. Somewhat similar features have been seen with the cells in tissue culture (fig. 7; *see* also the erythroblast from the circulating blood, fig. 3). In these micrographs of 2 different cells, there is plainly evident a sequence of processes representing the formation of particles by progressive budding at the cell membrane. The membrane thickens, then separates distally into 2 layers. Protrusion continues with narrowing of the pedicle and final separation of the particle.

In the extracellular space in figure 7B there are many virus particles showing the nucleoid surrounded, at low resolution, by a single limiting membrane. The particles still attached to the membrane and some of those free in the spaces show only a double membrane and are devoid of nucleoid. Measurements of the particles seen in figures 7A and 7B gave values of about 80 mμ for the diameters of the "incomplete" particles without nucleoid and for most of those with nucleoid. Some of those with the nucleoid, however, were of about 120 to 160 mμ diameter.

A variation of the budding process illustrated in figure 7 is apparent in figure 8. The picture shows a cell whose nucleus, cytoplasm, and mitochondria exhibit the same alteration evident in the cells illustrated in figure 4. At one site on the cell membrane at the *upper left*, budding similar to that already described occurs. In the cytoplasm of the cell there are 3 comparatively large vacuoles. The largest of these is centrally placed and bounded by a simple membrane free of Palade granules. Buds occur in the membrane directed toward the inside of the structure, and these have characteristics similar to those seen in the external membrane of the cell (fig. 7). In addition, vesicle-like structures occur in the lumen, the surrounding walls of which likewise exhibit the presence of the bud processes. From these characteristics it may be judged that the vacuole-like structures with the contained vesicles have been formed as the result of invagination of the cell membrane. The significance of the two other vacuolar structures is not entirely clear. Both, however, show some evidence of similar vesicular structures as well as internal amorphous material of indefinite character. It might be presumed that the vacuoles, though probably originating from cellular material, may be regarded as foreign bodies, and thus it may be assumed that the material within them is in the process of elimination by the cell. It might be assumed then that the content of the smaller vacuoles is constituted of deteriorating cellular material.

For comparison with the erythroblasts, a macrophage in a section of the spleen is pictured in figure 9. The features illustrated are characteristic of these phagocytic cells often encountered in the bone marrow and spleen of the chicken diseased with myeloblastosis, erythroblastosis, or lymphomatosis viruses (*25*). The cell contained large numbers of vacuolar structures, some of which were packed with virus particles, while others contained fewer particles in association with ill-defined, poorly stained, amorphous material. Some smaller, globoid bodies look like possible deteriorating virus particles. At the lower right is a similar structure with an enclosed, large, oval body having many deeply stained, small particles which may be deposits of iron conjugates (*26*). In the same body there are also seen virus particles and a filamentous arrangement suggesting remnants of cristae which may be either the remains of an ingested mitochondrion or protein precipitate. Some virus particles may lie free in the cytoplasm, but it is possible that they are situated instead in vacuoles whose membranes are not clearly discernible.

This appearance of the cell is similar to that which would be expected to be the result of phagocytic activity. This possibility was investigated, as in the erythroblasts previously described, by the injection of India ink into chickens several days after inoculation of virus. Large deposits of ink particles were observed enclosed in structures within the macrophages in an arrangement indistinguishable from that seen in figure 9. In addition, an occasional virus particle lay among the India ink particles. Thus, the picture shown by the cell in figure 9 was most likely the result

of phagocytosis, not only of virus particles but of structures from deteriorating cells taken up by the macrophages.

In some erythroblast cultures cells of the characteristics of macrophages occurred not infrequently. An example of these elements is illustrated in figure 10 in which the cell contains numerous large and small vacuoles, in some of which one or several typical virus particles are apparent. The origin of the phagocytic cells of this type has not yet been determined. Although it is conceivable that the cell is an altered erythroblast, the general features are those of a macrophage. There are a few intracellular virus particles in small, otherwise clear vacuoles. The structures designated as (MB) were not identifiable.

Myeloblasts in cultures thriving for periods up to 3 years have not evidenced significant changes in morphology. This has not been observed, however, with erythroblasts, which may undergo major alterations within a few weeks in culture, as indicated especially by the development of large numbers of vacuoles of various sizes, some of which occupy a large part of the cell. We also noted development of myelinated structures like those seen in figure 6A. The continued formation and liberation of virus into the culture fluid were demonstrable in thin sections. In experiment 10458, for example, pictures essentially identical with those of figure 4 were obtained at the 125th day of culture. Alterations in the distribution and margination of chromatin were not infrequent, since pictures similar to that of figure 4 were not uncommon.

DISCUSSION

The principal purpose of the work described in this report was not to consider in detail the general phenomena of tissue culture of the erythroblasts of avian erythroblastosis. Rather, the major aim was to investigate the ultrastructural characteristics of these cells in tissue culture with the hope of learning more about the principles of cell-virus relationships than was evident from analogous studies (*13–17*) of material obtained directly from the chicken. A prerequisite was the availability of cultures of growing erythroblasts in which it could be unequivocally demonstrated that the specific virus was being released by the cells. The results clearly demonstrated that these requirements have been fulfilled and that, although the conditions have not yet been found for uniform control of the growth of the cells, the findings may be utilized for further clarification of the virus-cell relationship. The results extend greatly those of investigators (*27–29*) working earlier with this disease.

From the actual problem of cultivation of erythroblasts *in vitro*, several conclusions may be drawn. Of first importance are the definite indications that the erythroblast in its response to the erythroblastosis virus possesses inherently the capacity for growth in tissue culture. The difficulties encountered with these cells are comparable with those ex-

perienced in the early work with myeloblasts. In the latter, the problem was resolved largely by recognition of the unusual requirements of the cells, not only for high concentrations of chicken serum, but especially of the dependence on folic acid for continued growth. The culture medium so useful with the myeloblasts still fails to support growth of erythroblasts, due either to lack of balance of nutrients and growth factors or to deficiencies not yet fully defined. More recent studies with cultures exhibiting greater uniformity in growth rates of cells give great promise for resolution of this problem.

Principally in comparison with the findings with myeloblasts, the ultrastructural characteristics of cell-virus relationships in erythroblastosis have been most remarkable. In the examination of material from the bird, the results of earlier workers (*13–17*) have been largely confirmed, but differences in detail, together with the experiments with cells from tissue culture, have led to interpretations at some variance from the concepts of other investigators.

For first consideration is the general observation of virus involvement of cellular elements other than the specific neoplastic cell, the erythroblast, in the diseased bird. These were primarily macrophages and reticular cells in the spleen and bone marrow, and endothelial cells in these tissues and in the liver. It is characteristic that these cells, particularly the macrophages, frequently contain enormous numbers of virus particles in masses segregated within well-delineated structures. The presence of virus naturally suggests a possible specific participation of these cells in the infectious process, and, indeed, it has been postulated (*9*, *14*) that some of these structures represent foci of virus synthesis possibly in altered mitochondria. It has been thought (*15*, *16*), too, that "incomplete" virus shed by erythroblasts may be taken up and synthesis completed in the macrophages.

None of the findings, however, supports the postulate that these cells actually contribute specifically to the disease in the sense that the virus might be biochemically integrated with the cell. In the first place, the cells are a well-recognized part of the reticuloendothelial system and are notably phagocytic. In consequence there is no reason to discount the likelihood that the cells would be as avidly phagocytic for the virus as for other particles of the same size. Second, it has been shown that the presence of erythroblastosis virus in these cells (*13–17*) is a phenomenon which occurs in the same way as in the diseases caused by the myeloblastosis, BAI strain A, (*6*, *30*) and lymphomatosis, RPL12 strain, (*25*) viruses. Third, the structures containing the virus exhibit scant evidence of organization of internal material, since the filament-like arrangements, seen in the present and earlier work (*9*, *14*) and thought to represent altered mitochondrial cristae, could constitute just as well ingested amorphous material or even mitochondria from other degenerated cells. It is noteworthy that such an appearance is rarely seen. Fourth, particulate material, India ink, introduced into the diseased bird is concentrated in the same cells in the same way, and such aggregates may contain virus

particles together with those of India ink. Finally, contrary to earlier implications (*17*, *25*, *30*), these cells *certainly do not exhibit neoplastic response* to the virus; *i.e.*, there is no evidence that the cells increase more in the diseased than in the normal chicken. Another point of probable significance was the observation that the virus particles in the intracellular structures had the same appearance as that of the particles of mature form, which was entirely unlike the form of the apparently incomplete particles budding at the cell membrane. For these reasons there is no basis for regarding the cells as "tumorous." There is no positive evidence that the cells are specifically involved; on the contrary, consideration of the absence of specific reaction and the well-known function of the cells suggests strongly that they participate only incidentally in the process and that the virus has reached the inside of the cell by simple phagocytosis.

That the cell-virus relationships with the erythroblasts are more specific and significant than those with the macrophages and reticular cells is unequivocally well grounded. As in the instance of the myeloblasts of myeloblastosis, virus was seldom seen in association with the erythroblasts in the circulating blood of chickens with erythroblastosis. In great contrast to the phenomena of myeloblastosis (*9*, *10*), however, the relationship of virus with the erythroblasts was altered very little, if at all, in tissue culture. Two types of response by erythroblasts are clearly evident, whether in the bird or in tissue culture: The first and most easily demonstrated is that of phagocytosis, a most surprising behavior of cells destined normally to become erythrocytes. That the cells possess this capacity was suggested immediately by the character of the structures containing accumulations of virus particles as illustrated in figures 3 and 5. The virus particles inside the cell exhibited the membrane and nucleoid characteristic of the agent in purified preparations (*23*). Furthermore, the material associated with the virus in the structures looked like amorphous extracellular material, and, finally, the ingestion of India ink particles by erythroblasts in the circulation and in tissue culture established their pronounced property of phagocytosis.

It has been suggested by others (*14*) and in an earlier report from this laboratory (*9*) that virus particles were seen in structures resembling altered mitochondria. In view of further information, it has been concluded that this hypothesis is not supported by the experimental findings and that the critical relationship concerned with virus synthesis is of an entirely different sort.

There seems no reason to doubt that the "budding" changes associated with the cell membrane, which were observed with relative frequency, represent the best morphologic evidence of synthesis of the erythroblastosis virus in association with the erythroblasts. These structures seen in the present study are somewhat similar to those reported by Benedetti and Bernhard, though their interpretations have been modified. It has been repeatedly observed here that the initial change associated with budding consists of an increase in density of a locus in the membrane which protrudes progressively and becomes underlaid by a second layer of dense

material. As protrusion proceeds, two concentric rings arise, well-separated, distinct, and symmetrical. In the final stage the separate rings become complete, the pedicles attaching them to the cell surface become increasingly constricted, and finally the particles, still showing clearly the double-ring characteristics, drop free of the cells.

The presence of 2 forms of particles, those with nucleoid and those without, raises the question of relationship, *i.e.*, whether the particles are different viruses or whether they represent different stages in the development of a single entity. There are several reasons for assuming the last alternative: 1) In all clear micrographs, the "bud" always exhibits the ring structure and the nucleoid is not seen; 2) the culture fluids, as well as the plasma of the diseased chickens, contain erythroblastosis virus of typical influence on the host; 3) there has been no evidence of a second viral agent; 4) thin sections of concentrates of the culture fluids and blood plasma reveal none of the double-ring structures; and 5) there is no evidence now to reveal any possible site of direct primary formation of particles with the nucleoid. For these reasons and in the absence of direct evidence to the contrary, it seems acceptable to regard the buds as the formative stages in the synthesis of the erythroblastosis virus. It is then necessary to conceive of the origin of the nucleoid as a condensation and probable alteration of the substance of the inner ring after separation of the particle from the cell. There is some direct evidence for this since, in the particles containing the nucleoid, the inner ring, as such, is no longer seen. In high-resolution pictures (*23*) there are traces of a possible double-wall membrane, but it is certain that the clearly defined inner wall in the double-ring structure cannot be seen.

That the avian leukemia viruses might be synthesized in, or in association with, mitochondria was an attractive hypothesis, but the positive evidence supports only the view that erythroblastosis virus is formed at the cell membrane. In this process it would appear that the initial morphologic stage consists in focal *alteration* of the cell membrane under the influence of determinative material in the cytoplasm beneath. Subsequently, the cytoplasmic material is likewise altered and becomes arranged as the second ring. That the cell membrane participates is certain, but the manner is unknown. It has been supposed (*14*) that certain vesicle-like structures often lying near the buds may constitute the material of the viral precursor. This possibility seems remote, since such bodies are common elsewhere, not only in the diseased but the normal cell as well. Furthermore, they are plentiful in the myeloblasts in which the virus is not formed at the membrane. A point of some importance is the assumption that the contribution of the cell membrane may confer host antigenicity on the virus. This is not indicated, since the changes in the membrane that are evident morphologically may well result in alteration of antigenicity.

It is of interest that the evidence of the site and the mode of synthesis of this avian leukemia agent at the cell membrane should be so similar to that observed not only with the agents of such widely varied tumors

as the mouse mammary carcinoma (*31*) and the Friend mouse leukemia (*32*), but also with nontumor viruses, as exemplified by the agents of fowl plague (*33*) and influenza (*34*). In extreme contrast is the situation in myeloblastosis (*9, 10*) in which the virus is synthesized in walled bodies and is eliminated from the cell without membrane involvement. Still different mechanisms are operative in the Rous sarcoma (*24*) in which definite evidence for participation of the cell membrane is lacking. Furthermore, although suggestive cytoplasmic and nuclear changes are observed in the cells of this disease, the regions of possible viral involvement are diffuse rather than enclosed. In view of these very marked differences in host response to the viruses of myeloblastosis, erythoblastosis, and Rous sarcoma, it should be recalled that the agents are members of the same family of interrelated viruses (*4*).

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PLATES

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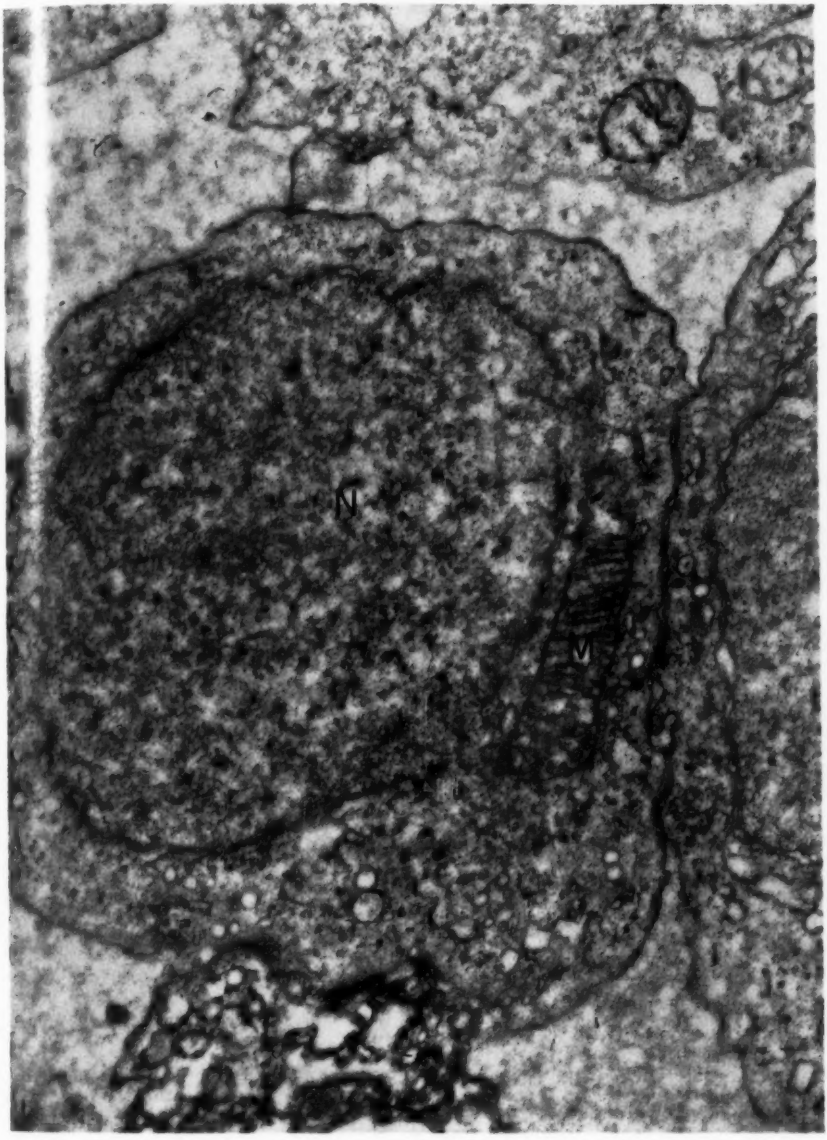

FIGURE 1.—Blast cell of the characteristics of an erythroblast observed in bone marrow of a normal chicken. Nucleus (N) is relatively large and cytoplasm sparse. No specific identifying features are evident. (M) = mitochondrion. × 22,500

FIGURE 2.—Blast cell like that of figure 1 from bone marrow of chicken with erythroblastosis 9 days after inoculation with virus. A few virus particles (VP) lie in extracellular spaces. No features of the cell exhibit evidence of specific association with the disease. (N) = nucleus; (M) = mitochondria; and (L) = lipide body. × 22,000

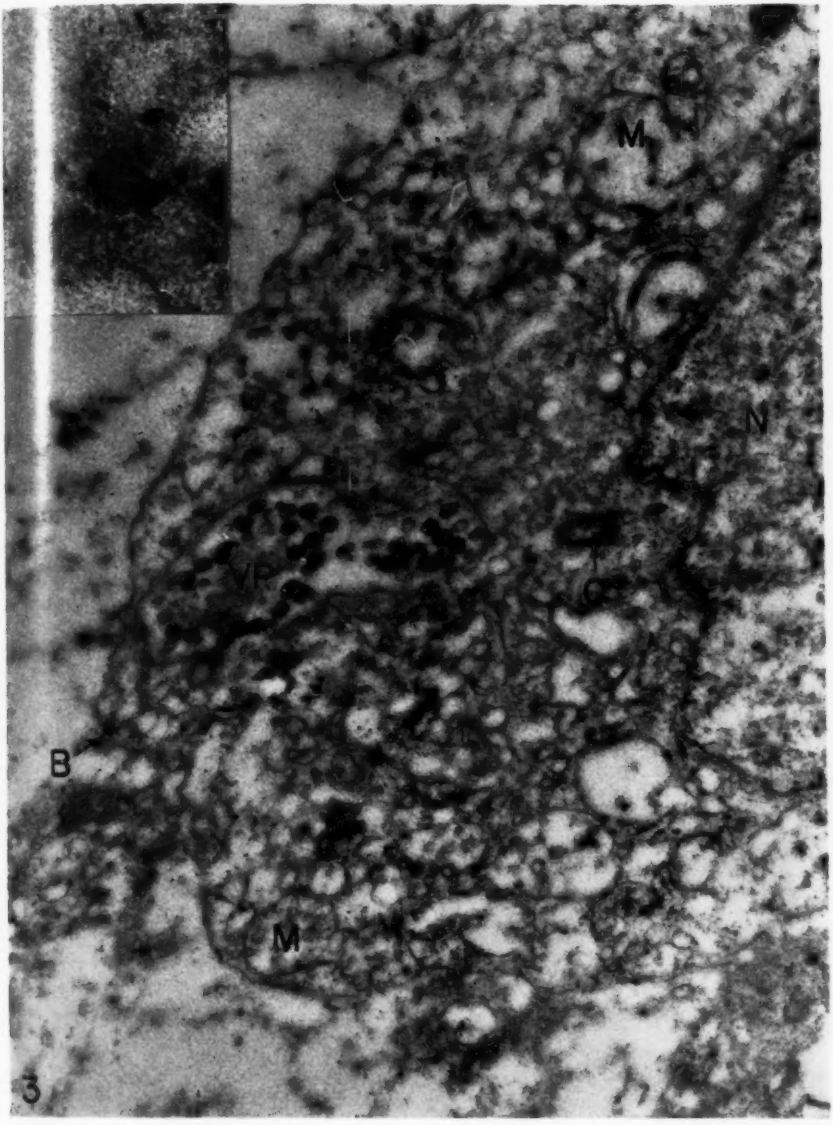

FIGURE 3.—Erythroblast from circulating blood of same chicken as shown in figure 2. General aspects of cell structure are typical of circulating neoplastic cells. A very infrequent feature is the large vacuolar structure containing many virus particles (VP) and much material with appearance of amorphous debris (*see* fig. 4). (M) = mitochondrion; (B) = virus bud (*see* fig. 6); (N) = nucleus; and (C) = centriole. × 30,000

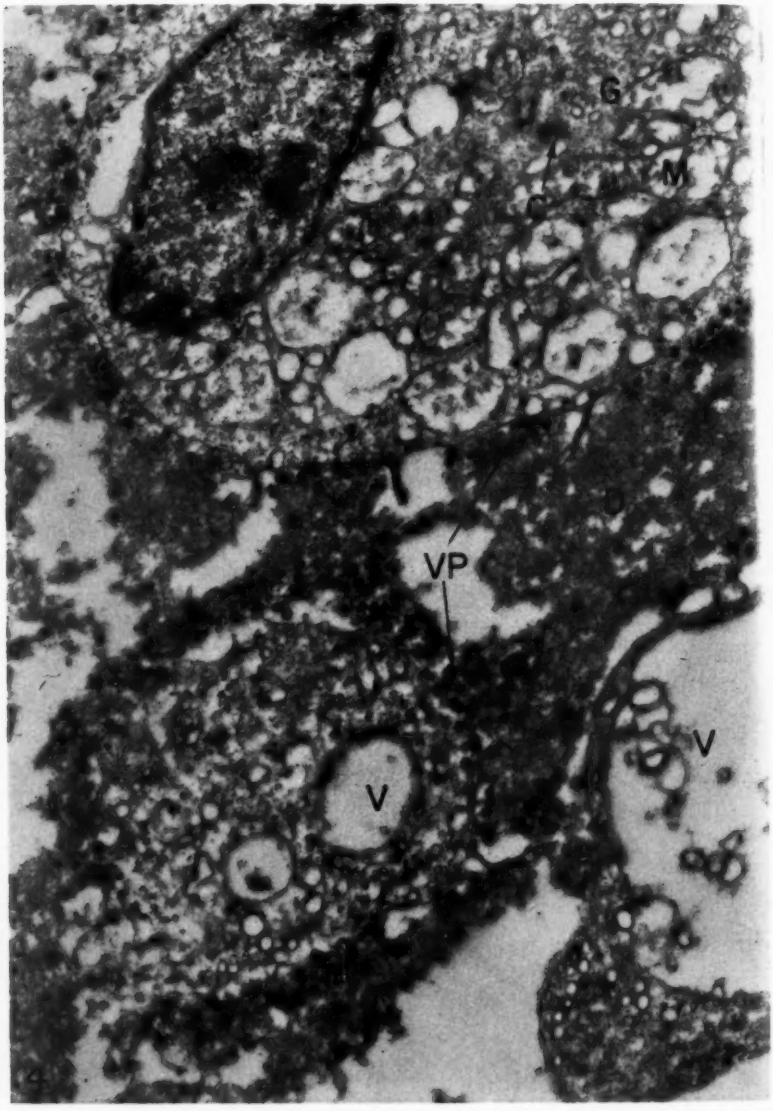

FIGURE 4.—Portions of cells from an 11-day tissue culture, (experiment 260, *see* text), of erythroblasts illustrating frequent occurrence of large numbers of virus particles (VP) accumulated near cell surfaces. Much extracellular material (D) of the appearance of debris is present. There are many protrusions of cytoplasmic membrane, mitochondria (M) are swollen, and cells often contained many vacuoles (V), some of which were very large (*lower right*). (M) = mitochondrion; (G) = Golgi region; (NU) = nucleolus; and (C) centriole. × 22,500

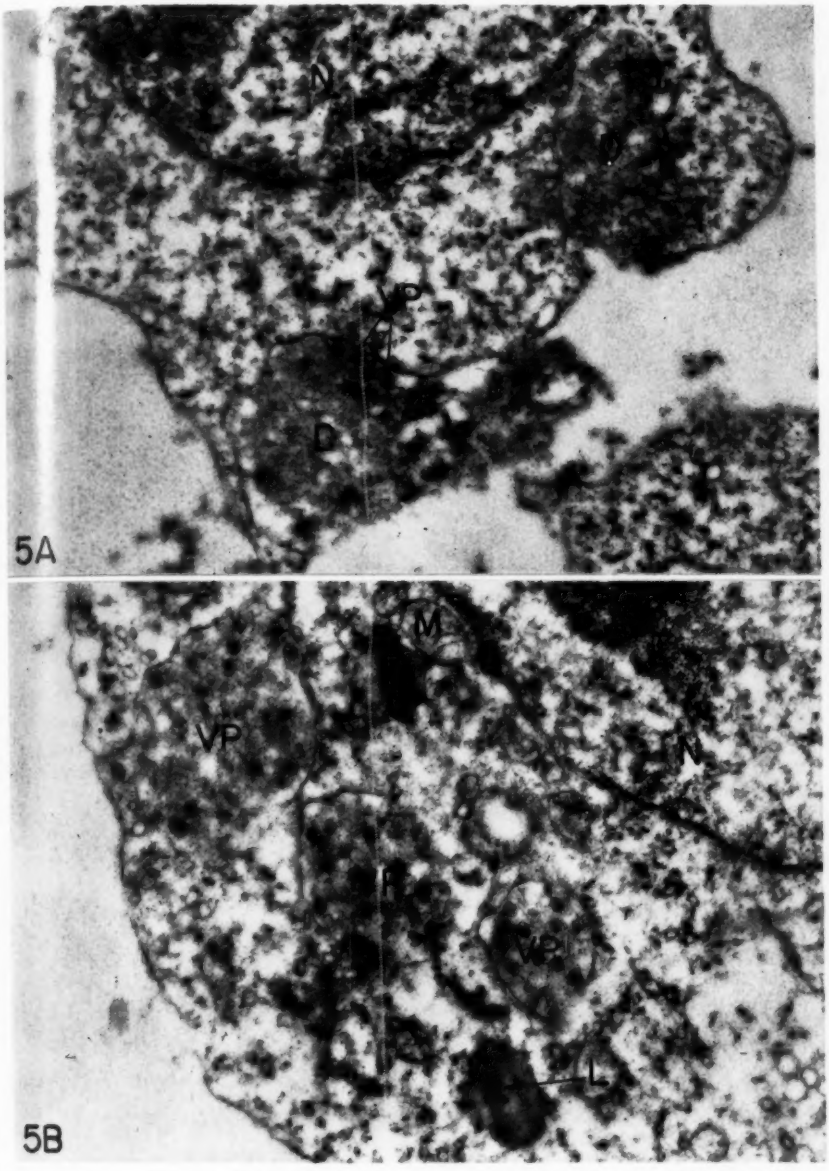

FIGURE 5.—Portions of 2 erythroblasts from a 2-day tissue culture (experiment 260, *see* text) suggesting phagocytosis of virus particles (VP) and associated extracellular debris (D). In figure 5A material (D) in which virus particles (VP) are embedded appears to be in process of engulfment, and in figure B, it is entirely within the cell. (N) = nucleus; (M) = mitochondrion; (NC) = nucleolus; and (L) = lipide. × 30,000

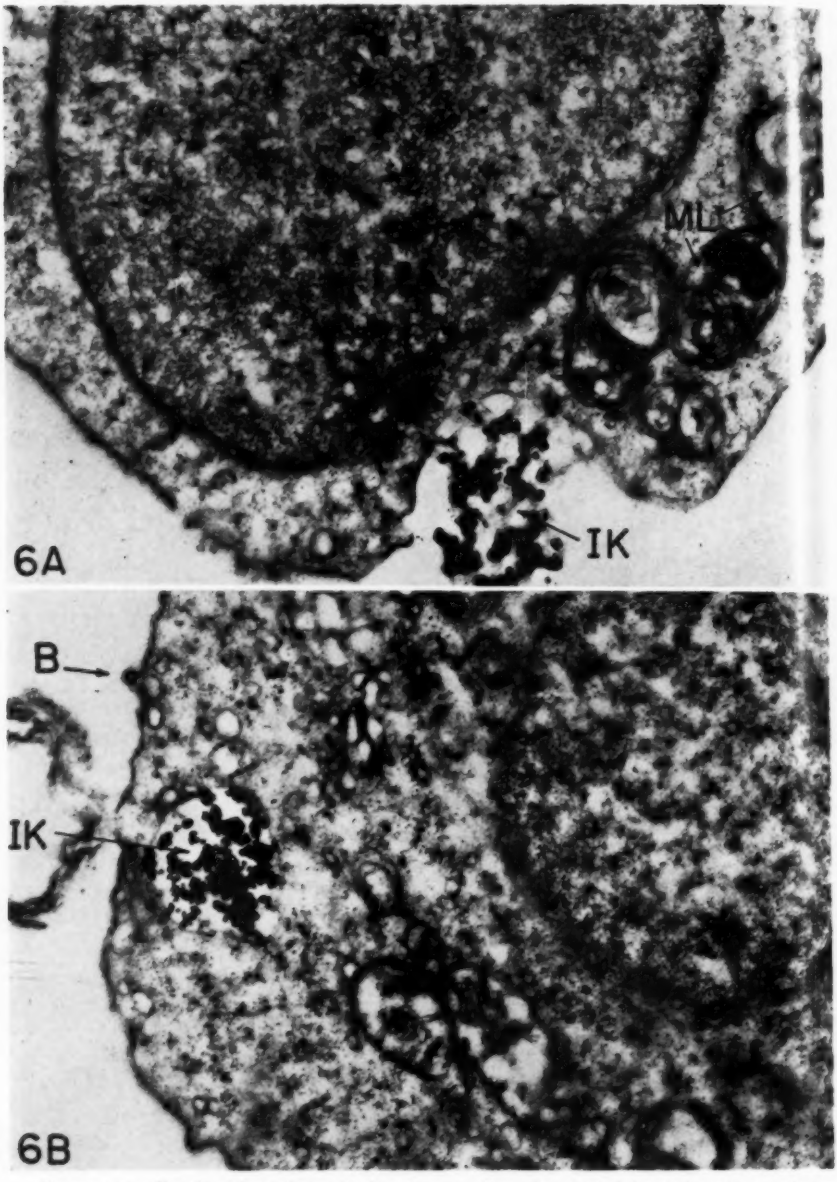

FIGURE 6.—Erythroblasts from 80-day tissue culture incubated for 2 days in routine culture containing 1 percent India ink. Figure 6A shows beginning engulfment of carbon particles (IK); in figure 6B this process has been completed and carbon particles (IK) lie in vacuole in cytoplasm of cell. (ML) = myelin structure. × 30,000

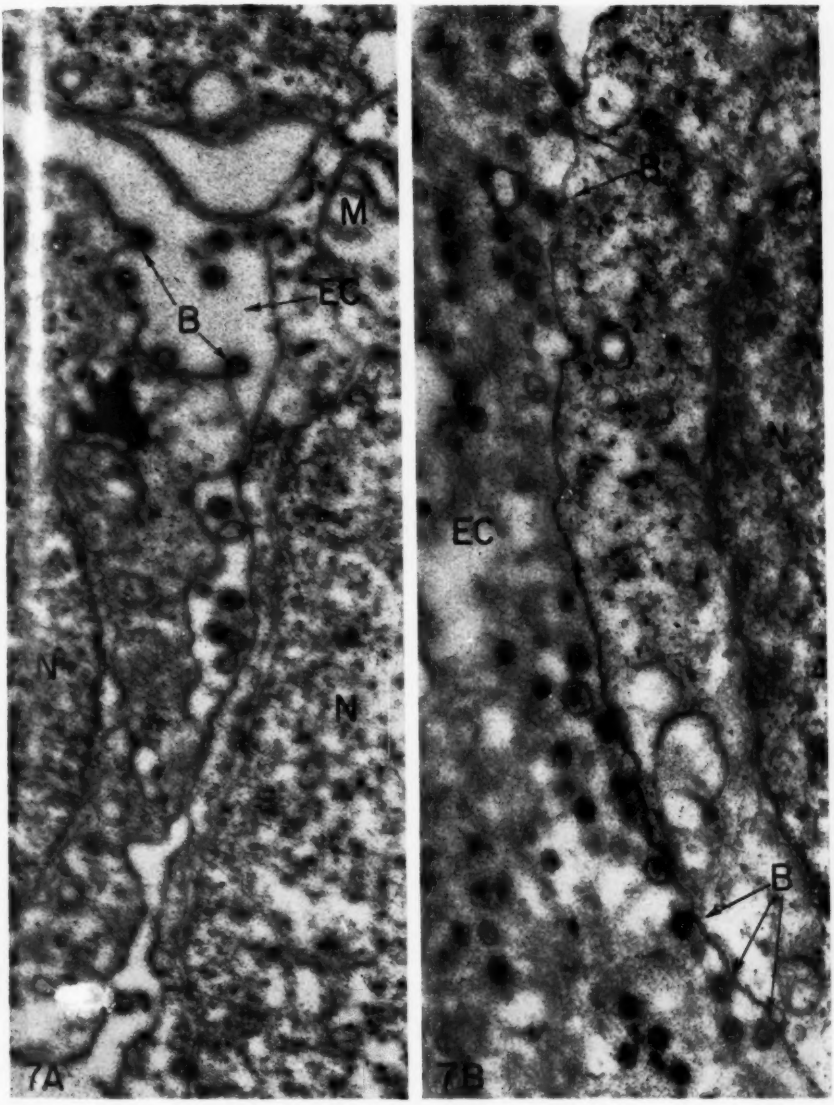

FIGURE 7.—Erythroblasts from 2 different 2-day tissue cultures, experiments 10458 and 260, illustrating appearance of different stages of budding (B) of virus particles from cell membrane. Particles in extracellular spaces (EC) vary from typical spheroids containing nucleoids to images with central areas of low contrast. (M) = mitochondrion and (N) = nucleus. × 50,000

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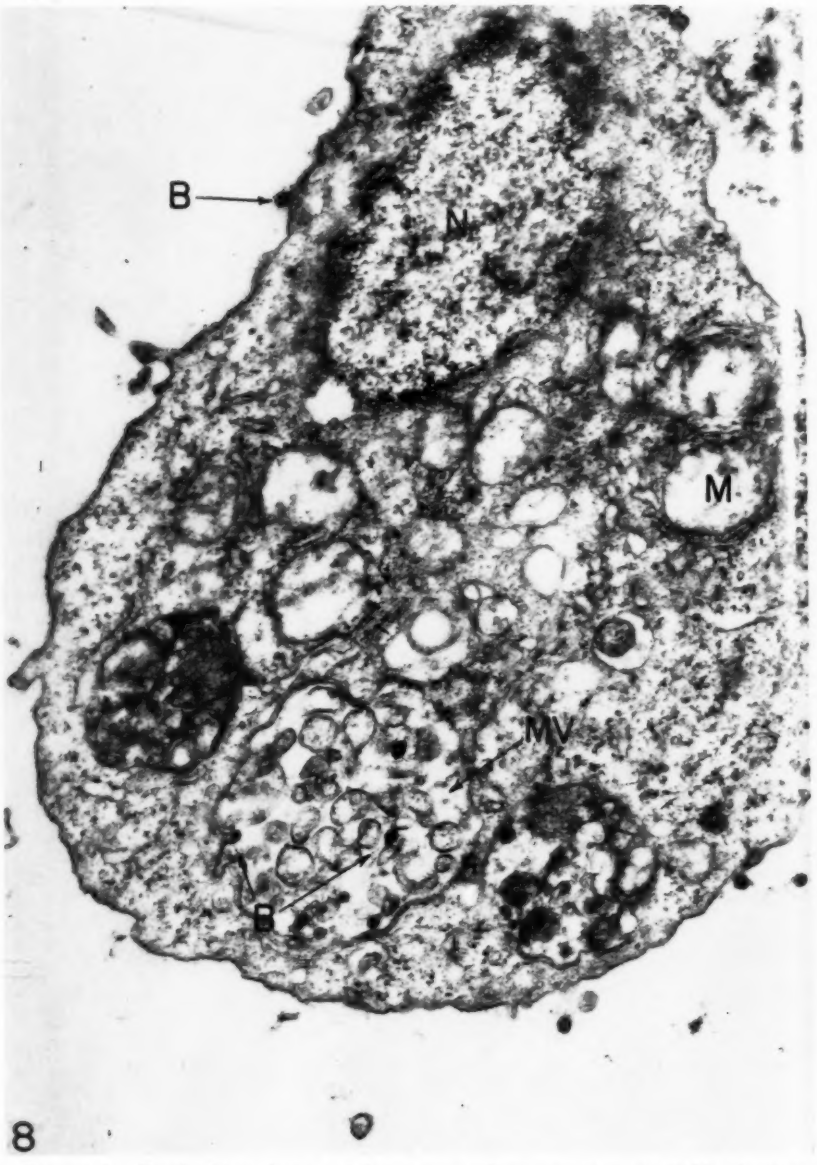

FIGURE 8.—Erythroblast from a 2-day tissue culture, experiment 10458, showing structures occasionally seen in cells *in vitro*. These have appearance (MV) of sections of possibly infolded cell membranes. Virus particles are seen budding (B) from these membranes and from external cell membrane (*upper left*). (N) = nucleus and (M) = mitochondrion. × 30,000

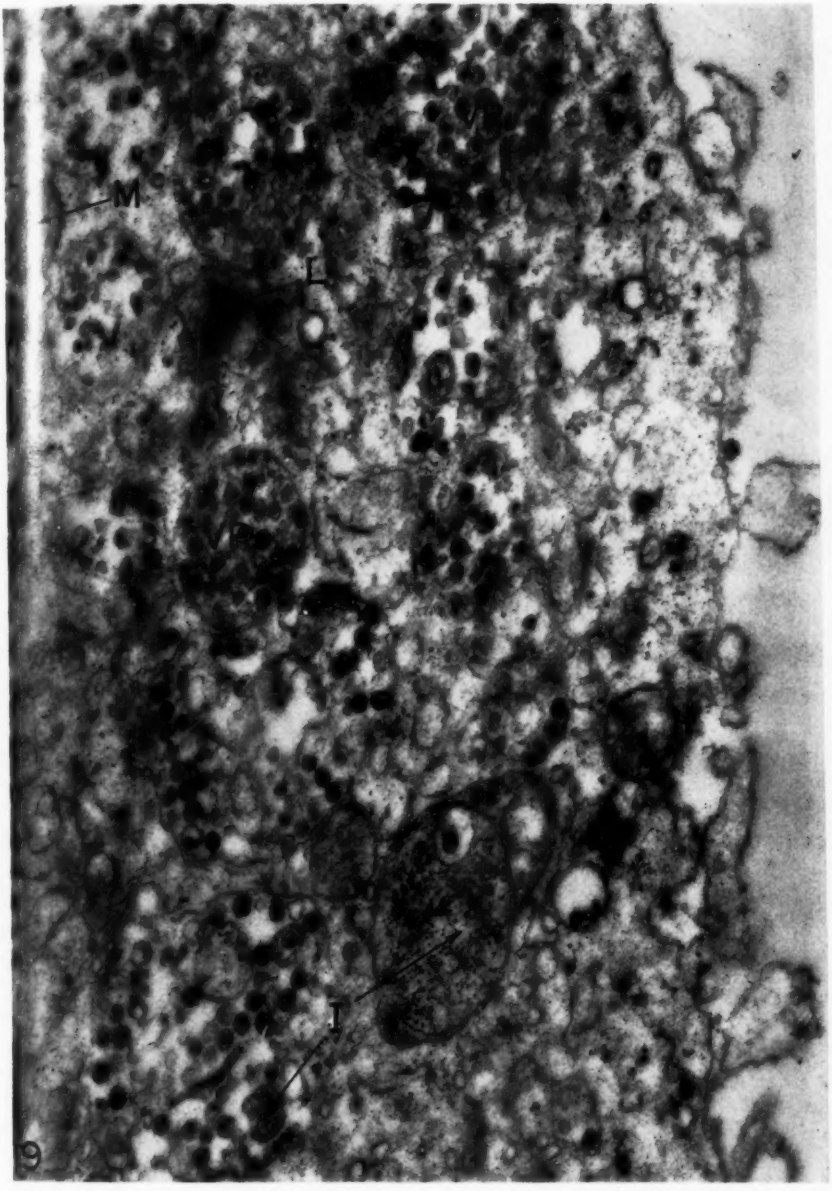

FIGURE 9.—Macrophage from bone marrow of chicken 7 days after inoculation with medium from 22-day tissue culture of erythroblasts. Many vacuole-like structures are packed with virus particles (VP) of typical features. In several (I) there are deposits of iron derivatives. (M) = mitochondrion. × 36,000

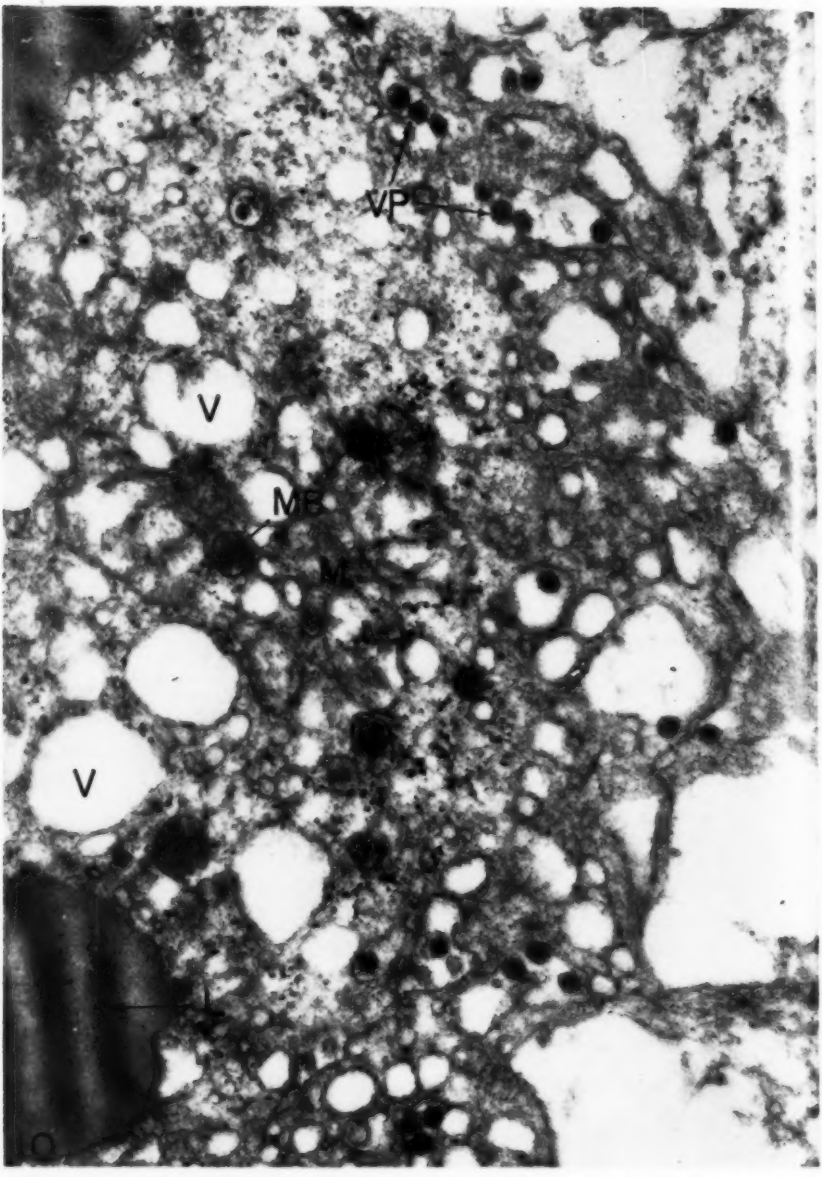

FIGURE 10.—Macrophage from a 7-day tissue culture prepared from circulating blood of chicken with erythroblastosis. Scattered virus particles (VP) of typical aspects are enclosed in clear vacuoles near cell periphery. Other bodies of uncertain nature (MB) bear some resemblance to microbodies (*see* text). (M) = mitochondrion and (V) = vacuoles. × 45,000

Studies on the Thymus of the Mammal. XII. Histochemistry of the Thymuses of C57BL/6 and AKR Strain Mice [1, 2]

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SUMMARY

The cortex of the thymus of young AKR strain mice was characterized by a generalized distribution of alkaline phosphatase activity. In unfixed, frozen sections cut in the cryostat, in addition to the reactive arterial endothelium, epithelial and mesenchymal reticular cells and lymphocytes were positive. In contrast, the C57BL/6 cortical cells were mostly negative except for scattered reticular cells and the endothelium. The medulla of thymuses from both strains contained strongly reactive oval cells. These cells were not as prominent in the medulla of strain AKR because it was populated with more reactive cells than in the medulla of strain C57BL/6. Focal areas of deeply colored cells also distinguished the AKR medulla. The cortex and the corticomedullary zone of the AKR thymus were usually characterized by many cells that were browned with the acid phosphatase technique; the medulla was almost devoid of positive cells. With the same technique, the C57BL/6 thymus was distinguished by a medulla and a corticomedullary region with scattered positive cells; the cortex was mostly negative. The thymuses of strain C57BL/6 retired breeders, though more or less involuted, were recognized by a pattern of alkaline phosphatase activity similar to that of the young mice. Those of strain AKR mice varied according to size, whether involuted or hyperplastic, and activity. Approximately half of the lobes of these thymuses from old AKR mice were more strongly reactive than the thymic lobes from young mice. In the 5-week-old mice, thymuses from the male and female AKR mice were heavier than those of the C57BL/6. Thymuses of the males of both strains weighed less than those of the females.—J. Nat. Cancer Inst. 26: 389–403, 1961.

A HISTOCHEMICAL study of the thymuses of 1) 4- to 5-week-old and 2) retired breeders of C57BL/6 and AKR strains of mice was undertaken because these two strains differ in their susceptibility to the development of spontaneous leukemia. In the highly leukemic AK strain, the thymus is primarily involved (*1*). Distinct differences between the thymuses of the two strains were found with the techniques for the demonstration of alkaline and acid phosphatases. In unfixed, frozen sections, cut in the cryostat, lymphocytes in the AKR thymus contained

[1] Received for publication August 8, 1960.

[2] Supported by research grant C-1553(C8) from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] The author is indebted to Sandra J. Durick for technical assistance.

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alkaline phosphatase, whereas those of the C57BL/6 did not. Also the medulla of the AKR thymus was characterized by foci of cells much more reactive than any others. With the acid phosphatase technique, the cortex and the corticomedullary zone of the AKR thymus were much more reactive than the medulla; in the C57BL/6 thymus, the medulla was characterized by numerous scattered positive cells and the cortex usually by few.

MATERIALS AND METHODS

Mice, 4 to 5 weeks old (C57BL/6, 6 males, 8 females, and AKR, 6 males, 6 females) and retired breeders, 8 to 11 months old (C57BL/6, 7 males, 13 females, and AKR, 18 males, 14 females) were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The mice were killed by cervical dislocation, and thymuses were removed, frozen on dry ice, and stored in a deepfreeze at −18° C. Sections were cut routinely at 10 μ (some at 5 μ) in the cryostat at −18° C. For each technique some thymuses from C57BL/6 and AKR mice were incubated in substrates in the same Coplin jar.

For the demonstration of nonspecific *alkaline phosphatase* and *adenosinetriphosphatase*, Gomori's glycerophosphate-cobalt sulfide methods (*2*) modified by Padykula and Herman (*3*) were used. Unfixed, frozen sections were dried in front of a fan for ½ hour, stored no longer than 3 hours at 4° C, and incubated for the following lengths of time: β-glycerophosphate, 2 hours; adenosine triphosphate, 10 minutes. In addition, an α-naphthyl phosphate-azo-dye method for the demonstration of alkaline phosphatase was used (*4*). These sections were incubated at 4° C for 1 hour (to diminish the formation of bubbles which appeared when incubated for ½ hour at room temperature) and immersed in acid alcohol (1% HCl in 50% alcohol) to remove any background staining and remaining bubbles. Sections incubated at 4° C for 15 or 30 minutes were similar in pattern to those incubated for a longer period except that the reaction was not as strong. The azo-dye method gave a clearer picture of the thymus because the nuclei were unstained and the sites of slight activity in the cytoplasm were indicated by distinct, fine black granules of the precipitated dye. This material was examined as soon as possible after preparation. The two methods were always checked against each other. To show sites of *acid phosphatase* activity, frozen sections were cut, dried in front of a fan for ½ hour and incubated for 2 hours according to the glycerophosphate-lead sulfide method of Gomori, according to Lillie (*5*). Safranine and hematoxylin were used as nuclear stains for some of the sections.

At intervals in the series, sections were cut at 5 μ, dried in front of a fan for 2 minutes, fixed in fresh Carnoy's solution for ½ hour, and stained in May-Grünwald-Giemsa's solution (*6*).

Control sections were inactivated by immersion in boiling water for 5 minutes.

To aid in the interpretation of the unfixed, frozen sections, thymuses from additional C57BL/6 and AKR mice (5 weeks old, 3 of each strain) were divided transversely into 3 parts. One portion was fixed in ice-cold acetone and embedded in paraffin (*7*); one in formal-calcium embedded in gelatin (*8*); and the third was unfixed, frozen, and cut in the cryostat. The formal-calcium and unfixed, frozen sections were stained in Oil Red O. Larger thymuses survived this treatment but smaller ones from the C57BL/6 mice were so damaged by handling that data from them could not be used. Other thymuses (2 of each strain) were fixed whole in the cold acetone and embedded in paraffin. For alkaline phosphatase, we incubated this material for 4 hours using the glycerophosphate-cobalt sulfide method; 1 hour at room temperature with the azo-dye method; and for acid phosphatase, 12 hours with the glycerophosphate-lead sulfide method.

OBSERVATIONS

Histochemical Analyses of the Thymuses of AKR and C57BL/6 Mice 4 to 5 Weeks Old

A distinct strain difference was apparent between the thymuses of the AKR and C57BL/6 strain mice with the techniques for the demonstration of nonspecific alkaline and acid phosphatase activities. The distinguishing marks were so clear that thymuses (12 of each strain) chosen at random and presented as unknowns could be diagnosed accurately with both alkaline phosphatase techniques and in most cases with the acid phosphatase procedure.

The thymuses which were studied histochemically could not be weighed because the mediastinal lymph nodes, blood vessels, and fat were used for reference in the appraisal of the different techniques. However, the thymuses of AKR mice were obviously larger than those of the C57BL/6 and male thymuses appeared larger than female. Determinations of absolute and relative weights confirmed these observations (table 1). The differences between weights of the AKR and C57BL/6 thymuses were significant.

Alkaline Phosphatase

The endothelium of the medullary arteries and cortical arterial capillaries was characteristically blackened in both strains.

C57BL/6 thymus azo-dye technique

The thymus of the C57BL/6 mice (fig. 1) had the following characteristics: 1) a cortex composed mostly of unreactive lymphocytes and reactive reticular cells;[4] 2) a medulla more reactive than the cortex, distin-

[4] The main types of thymic cells are the epithelial reticular cells with large, oval vesicular nuclei, thin nuclear membrane and a bleblike nucleolus to which are attached one or more karyosomes; mesenchymal reticular cells with coarsely chromatic nuclei; and lymphocytes.

TABLE 1.—Absolute and relative weights of thymuses of C57BL/6 and AKR mice

Mice	Number of animals	Body weight (g)	Thymus weight (g)	Thymus weight (percent)
5 Weeks old				
C57BL/6 female	13	16.3 ± 2.37	0.052 ± 0.016	0.32
C57BL/6 male	12	18.6 ± 1.44	0.027 ± 0.015	0.14
AKR female	14	21.0 ± 2.36	0.117 ± 0.020	0.58
AKR male	14	21.3 ± 2.41	0.091 ± 0.028	0.43
Retired breeders*				
C57BL/6 nonpregnant female	8	34 ± 3.00	0.044 ± 0.018	0.13
C57BL/6 pregnant female	4	40 ± 3.08	0.038 ± 0.057	0.10
C57BL/6 male	10	31 ± 2.34	0.026 ± 0.072	0.08

*The weights of the thymuses of AKR retired breeders were not determined.

guished by sections of blackened arteries, scattered positive reticular and strongly reactive oval cells, negative lymphocytes except in the neighborhood of reactive cells and arteries. The nuclei were colorless. When sites of activity were present in the cytoplasm, black granules in the lymphocytes outlined the clear nucleus and brought out the processes of the mesenchymal reticular cells. In the epithelial cells the nucleus was usually outlined by the colored precipitate. A round, black inclusion marked the oval cells (fig. 1). As most lymphocytes with dye particles in their cytoplasm surrounded reactive arteries, reticular cells, or oval cells, the possibility of diffusion was considered. Loustalot (*9*, *10*) suggested that appearances of diffusion such as were present in the C57BL/6 thymus, particularly after the azo-dye technique, occurred after storage "due to continued enzyme activity on residual substrate" (*10*, p. 406). These storage artifacts were reduced following incubation "after brief treatment" with Lugol's solution. As shortened periods of incubation, lengthened periods of washing in running water, and the cutting of thinner sections did not prevent this appearance of diffusion in the thymus, we tried treatment with Lugol's solution (2 g of K iodide, 1 g of iodine, 200 ml of distilled water). The slides were immersed for 1 to 3 or 10 to 20 minutes in the iodine solution immediately after incubation with only a slight rinse in water or after washing for a longer period. The condition of the lymphocytes around the medullary arteries was the criterion for the efficacy of the treatment. No consistent result was obtained after treatment with Lugol's solution. With our usual azo-dye procedure the extent of diffusion around reactive arteries and cells also varied among the thymuses, and the conclusion was reached that the phenomenon took place during incubation.

Glycerophosphate cobalt-sulfide technique

With this procedure for the demonstration of sites of alkaline phosphatase activity, the characteristics of the thymus (fig. 2) were the same as those listed for the azo-dye technique except that sites of slight activity

were more difficult to see because the nuclei were colored, though not as strongly as in fixed sections. The extent of the medulla is shown by the looser arrangement of cells and by the presence of the dark arteries and black oval cells (fig. 2); no clear corticomedullary line is evident. Diffusion of the enzyme was also less noticeable than with the azo-dye technique because of the general tan color of the nuclei.

The details of the strongly reactive, typically oval cells of the medulla were more distinct with this procedure than with the azo-dye method (figs. 7 and 8). The tinted, eccentric round nucleus was usually characterized by coarse flakes of chromatin or a central nucleolus. Other similar cells had nuclei which were bean-shaped, elongated, or flattened (fig. 9). The site of activity within the cytoplasm was clearly delimited as a round, black inclusion which varied in size from smaller to larger than the nucleus. Under high magnification, this inclusion was seen to be composed of closely crowded, round granules (fig. 7) with black surfaces or granules embedded in a reactive cytoplasmic ground substance. In sections treated with the periodic acid-Schiff technique, cells similar in shape to the oval cells contained inclusions which stained a deep rose in undigested and digested material. Comparable cells in paraffin sections of thymuses fixed in Bouin's fluid or in frozen sections postfixed in Carnoy's solution and stained with May-Grünwald-Giemsa's solution had eccentric nuclei and pale lavender-pink cytoplasm. However, inclusions, irregular in shape and unequal in size, which stained pink in Giemsa's solution, were seen in thymuses which had been fixed in ice-cold acetone and embedded in paraffin.

AKR thymus, azo-dye technique

In contrast to the C57BL/6 thymus, the AKR thymus (fig. 3) was distinguished by the following features: 1) a cortex in which clusters of more reactive reticular cells and lymphocytes were seen among less reactive lymphocytes; 2) a medulla which was (*a*) similar, (*b*) more reactive, or (*c*) less reactive than the cortex; and 3) distinctive, strongly reactive areas within the medulla. Because the cortical cells of the C57BL/6 thymus contained so few sites of activity, the outer edge of the cortex was faint (fig. 1), whereas that of the AKR thymus was clear-cut (fig. 3). Blackened oval cells were also present in the AKR medulla (figs. 3 and 4), but were not as prominent as in the C57BL/6 because they were surrounded by other reactive cells.

Glycerophosphate cobalt-sulfide technique

With this method, the darkening of the whole thymus was intensified by the coloring of the nuclei; the corticomedullary line was often more distinct. Cortical and medullary lymphocytes contained dark brown or black granules; these were most prominent in the reactive areas of the medulla. In figure 4, although an artery is present at the center of the reactive region of the medulla, the deepened color was not considered to be caused by diffusion, since many other areas near reactive arteries were not similarly stained.

Acid Phosphatase

With the glycerophosphate-lead sulfide method for the demonstration of nonspecific acid phosphatase in the C57BL/6 thymus, the mesenchymal reticular cells, located mostly in the medulla or corticomedullary zone and only thinly scattered in the cortex, were dark brown (fig. 5). In contrast, the usual pattern in the AKR thymus was a cortex and corticomedullary region sprinkled with numerous more strongly reactive cells and a medulla which contained few positive cells (fig. 6). Although the difference between the two strains was generally evident with this technique, there were AKR thymuses, or portions of them, in which the distribution of sites of enzyme activity was similar to that of more reactive C57BL/6 thymuses.

Adenosinetriphosphatase

No differences were detected between the two strains with the technique for the demonstration of this enzyme. The endothelium of all blood vessels was characteristically blackened. In both, the medulla was somewhat more reactive than the cortex. Cells similar to the oval medullary cells, described as strongly reactive with the techniques for nonspecific alkaline phosphatase, were positive in these preparations.

May-Grünwald-Giemsa Stain

The preparations stained in May-Grünwald-Giemsa solutions were used for comparison of general structure. In both strains the thymus presented the usual characteristics of a cortex packed with small lymphocytes with deeply chromatic nuclei, some larger lymphocytes interspersed with mesenchymal and epithelial reticular cells, and a medulla composed of more loosely arranged lymphocytes and reticular cells.

Histochemical Analyses of the Thymuses of C57BL/6 and AKR Retired Breeders

The state of the thymuses of the retired breeders was more variable than that of the younger mice. The C57BL/6 thymuses had undergone more or less involution as evidenced by their reduced size. Chromolipoid or foamy cells characteristic of the cortices of older thymuses were present in almost all (*11*). Among the AKR mice, thymuses might be involuted to different degrees or greatly enlarged. In thymuses which had apparently become well-developed lymphomas, foamy cells were rare or absent.

Alkaline Phosphatase

The characteristics of the thymus of the C57BL/6 retired breeders were essentially those described for the thymuses of the younger mice, with the following exceptions: Among 20 mice observed in this investigation, the posterior border of the cortex of both lobes of one thymus contained moderately reactive lymphocytes. The rest of the cortex was

more like that of a young AKR thymus than a C57BL/6. One lobe of another thymus (in a series not a part of the present study and in a mouse irradiated 24 hours previous to being killed) was partially composed of cells reactive with the alkaline phosphatase techniques.

The cortices of thymuses from the older AKR mice were either similar to those of the 4- to 5-week-old mice, or one or both lobes were much more strongly reactive; among 14 females, 7 were more reactive, among 18 males, 10. The thymus from a male mouse pictured in figure 10 was estimated to be nearly normal in size. One lobe was much more reactive than the other (*cf.* fig. 3). Involuted thymuses were more frequent among the males in this series. The lobes varied from normal to strongly reactive. Figure 11 is a photograph of an involuted thymus in which the cortex of one lobe was intensely reactive and the other only moderately so. Among the thymuses of the females, the larger lobe was usually slightly or much more reactive than the smaller lobe. An example of intense activity in a lobe judged to be 3× the normal size is shown in figure 12. The increased reaction of the smaller lobe can be compared with its paler medulla. The uneven appearance of its cortex with clusters of more reactive cells shows clearly. However, the more reactive lobe may be smaller (fig. 13). In this thymus the larger lobe was unreactive, one of two instances in this series of 32 mice. When the lobes of these thymuses differed in intensity of reaction, there was no diffusion from the stronger to the weaker (figs. 10, 12, and 13).

Acid Phosphatase

Whereas some thymuses of the C57BL/6 retired breeders resembled those of the younger mice in the pattern of acid phosphatase activity (a more reactive medulla), many possessed so few positive cells that the sections could hardly be seen if no nuclear counterstain were used. In sections of AKR thymuses taken through on the same day, the many strongly reactive cortical cells characteristic of this strain were well demonstrated. The cortex of the C57BL/6 thymus which possessed reactive lymphocytes, with the technique for alkaline phosphatase, was also unique with the procedure for acid phosphatase; many positive cells were present. Well-developed lymphomas in the AKR mice were characterized by scattered reactive cells all through the section. Statements concerning acid phosphatase activity are to be found in the legends for figures 11, 12, and 13.

Adenosinetriphosphatase

The pattern of sites of activity of this enzyme in the C57BL/6 thymuses was the same as in the younger organs, the medulla more reactive than the cortex. Among the AKR thymuses there was great variation. In about half, the normal distribution was evident. In the rest, regions might differ within a lobe: Positive cells were sometimes scattered throughout, more evenly in some areas than others, with no relation to cortex and medulla. In large lymphomas the thymic vascular pattern was lost.

May-Grünwald-Giemsa Stain

The thymuses of the C57BL/6 mice appeared the same after they were stained as those of the younger mice. In most of the AKR thymuses, whether enlarged or not, the lymphocytes were of the larger type. The legends for figures 11, 12, and 13 include descriptions of the cells stained with May-Grünwald-Giemsa solution.

Lipides

Unfixed, frozen sections and sections from portions of thymuses fixed in formal-calcium and embedded in gelatin were colored with Oil Red O. In both strains lipides were present in scattered reticular cells, but few colored granules were seen among the lymphocytes.

Acetone-Fixed, Paraffin-Embedded Thymuses

The same general pattern of sites of alkaline phosphatase activity characteristic of the unfixed, frozen sections in the two strains of mice was seen in the young thymuses fixed whole in cold acetone and embedded in paraffin. More numerous and more reactive cells, seen best with the azo-dye technique, were distributed throughout the AKR cortex. In some regions a narrow, darker, subcapsular zone was differentiated. The cells were epithelial and mesenchymal reticular with some lymphocytes. The dark areas in the medulla were prominent. The sections treated for the demonstration of acid phosphatase activity did not show the differences noted in the unfixed, frozen sections.

DISCUSSION

In the course of histochemical studies, strain differences became apparent in thymuses of C57BL/6 and AKR mice in respect to nonspecific alkaline and acid phosphatases demonstrated in unfixed, frozen sections. In the AKR strain, thymic lymphocytes in both cortex and medulla contained alkaline phosphatase. In the medulla, localized areas of more strongly reactive cells were distinctive (figs. 3 and 4). [This condition of the medulla distinguished the AKR thymus from thymuses of other mice. In members of some other inbred lines of mice[5] both cortical and medullary lymphocytes were also positive with the techniques for alkaline phosphatase but lacked the distinctive, stronger foci in the medulla (unpublished data).] In contrast, the thymic lymphocytes of the C57BL/6 thymus were unreactive, except for those few in which response might be due to diffusion. This view is contrary to the opinion we expressed in 1958 (*12*) when, from our observations of thymuses of C57BL/6 mice treated with the Gomori cobalt-sulfide method, we believed that sites of activity could often be seen in cortical lymphocytes. It should be noted that

[5] The author is indebted to Dr. Kathryn F. Stein, Mount Holyoke College, for these inbred lines of mice.

lymphocytes which are positive with the alkaline phosphatase techniques (both glycerophosphate and azo-dye methods) have been observed in some thymuses of C57BL/6 mice previously exposed to staggered doses of X ray (unpublished data).[6]

The presence of alkaline phosphatase in lymphocytes has been debated for some time. Trowell (*13*) has probably expressed the prevailing opinion in his statement (p. 265) that "Lymphocytes contain no alkaline phosphatase. . . . The only alkaline phosphatase in lymphoid tissue is in the blood endothelium and this is probably adsorbed plasma phosphatase." However, Doyle (*14*) described the presence of alkaline phosphatase in reticular cells of the rabbit appendix, and Smith, Wharton, and Gerhardt (*12*) noted similar positive cells in the thymus and in certain regions of the spleen and lymph node. Indeed, Winquist (*15*) suggested that the azo-dye method might be specific for mesenchymal reticular cells of the bovine thymus.

The apparent presence of alkaline phosphatase in some lymphocytes in the C57BL/6 thymus might be explained as the result of diffusion from the reactive endothelium, reticular, and oval cells. It was noted by Doyle (*14*, p. 98) that "The staining reactions of the lymphocytes vary in intensity depending upon their location amongst phosphatase-rich or phosphatase-poor cells." As the appearance of diffusion from reactive arteries and cells was present in the thymus when incubated for as short a time as 15 minutes at 4° C (azo-dye method), and as no consistent results were obtained with the treatment with Lugol's solution, it was considered that, in the unfixed, frozen sections, this process took place during incubation and that the extent varied among thymuses. There was no diffusion between reactive lobes and adjacent less reactive or negative lobes.

In these unfixed, frozen sections, evidence of much more alkaline phosphatase activity in the AKR thymus than the C57BL/6 was consistently observed, both in the 4- to 5-week-old mice and the retired breeders. Only in 2 large lymphomatous lobes (fig. 13) was the reaction absent. It is possible that sites of enzyme activity are lost in old tumors. More numerous reactive reticular cells and lymphocytes in the cortex and medulla were the source of the general increase in the AKR thymuses. Pearse (*10*, p. 405) stated: "It is essential that the color of the azo dye final product should be as dark as possible and that the precipitate should be small . . . the final product precipitated in the tissues should have particles visible under high magnification. If the final product is not particulate, staining of lipide components of the tissue is likely to be indistinguishable, if it occurs, from the specific deposition of the dye." Although fine particles could be seen in lymphocytes under high magnification with both techniques for the demonstration of alkaline phosphatase, tests were run to detect the presence of lipides in the cells of the

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[6] The author is indebted to Dr. Henry S. Kaplan, Department of Radiology, Stanford Medical Center, Palo Alto, Calif., for the irradiated mice.

thymuses. In both unfixed, frozen, and the formal-calcium-fixed AKR thymuses, lipides were present in some reticular cells, otherwise observable only as a few scattered granules among the lymphocytes which were not enough to account for the appearance of the lymphocytes when colored by the azo dye.

A preliminary study was made of cold acetone-fixed, paraffin-embedded material to compare with the unfixed, frozen sections. As Pearse (*10*) stated only 30 percent of the alkaline phosphatase activity remained after such treatment, it was hoped that the loss of so much enzyme would leave behind those sites of greatest activity. The general pattern was the same in the fixed material of the 5-week-old mice as in the unfixed in both strains. In the acetone-fixed as well as the unfixed, frozen sections, the azo-dye method brought out the activity of the thymic cells more clearly. Winquist (*15*) also found this with the bovine thymus, though he preferred frozen-dried preparations. The sites of most activity which remained in the AKR cortex, in addition to the endothelium of the arteries, were epithelial and mesenchymal reticular cells and some lymphocytes. The strongly colored regions in the medulla retained their distinctive appearance. The C57BL/6 cortex possessed only reactive arterial endothelium and few reticular cells; more reactive cells were in the medulla. A study of the histogenesis of the AKR thymus to see whether any sequence in the development of sites of cellular activity could be discovered would be interesting.

Further study of the differences between the C57BL/6 and AKR thymuses with the technique for demonstrating sites of acid phosphatase activity needs to be done, since the preliminary survey of the fixed material failed to corroborate the patterns of activity which characterized the unfixed, frozen thymuses.

Another difference between the two strains was indicated by the greater weights of the thymuses of both male and female AKR mice compared with those of the C57BL/6 mice. These data confirm results obtained by Arnesen (*16*). In an investigation of strain differences between the leukemic AKR/O (Oslo strain) and the WLO (an albino strain with no cases of leukemia on record), Arnesen (*16*) made the following observations: a greater absolute and relative weight in both sexes in the AKR/O strain; marked overweight of the female AKR/O thymus; a smaller sex difference among the WLO's that disappeared in older mice; and a normal pattern of involution in the WLO thymus but in the AKR/O a preleukemic atrophy with hyperplasia of the epithelium.

Although alkaline phosphatase was demonstrated in the lymphocytes of the thymuses of young and old AKR mice, and although the enzymatic activity was stronger in these cells in many of the older thymuses, an interpretation of results cannot be made at this time. The focal sites of strong activity which distinguish the medulla in this strain may be important. At least, a general statement cannot be made that lymphocytes do not possess alkaline phosphatase; obviously under some conditions sites of alkaline phosphatase activity are present in thymic lymphocytes.

ADDENDUM

Since the completion of this paper, a preliminary examination has been made of thymuses from 3-month-old male C57BL/6 and AKR mice. At this age no strain differences were observed with the techniques for the demonstration of alkaline phosphatase. A comparative study of these two strains at different ages has been instituted.

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PLATE 52

Thymuses of 4- to 5-week-old C57BL/6 and AKR mice treated for the demonstration of alkaline (figs. 1 to 4) and acid (figs. 5 and 6) phosphatase. Unfixed, frozen sections, cut in cryostat at 10 μ. Green B58 filter. × 72

FIGURE 1.—C57BL/6 thymus—alkaline phosphatase, azo-dye technique. Arteries (a) and arterial capillaries (c) are *blackened*. No clear corticomedullary line (CM). Edge of cortex (C) is faint. Reactive oval cells (o) stand out clearly. Outlines of lymphocytes are caused by refraction; medulla (M).

FIGURE 2.—C57BL/6 thymus—alkaline phosphatase, glycerophosphate-cobalt sulfide technique. *Same thymus* as figure 1.

FIGURE 3.—AKR thymus—alkaline phosphatase, azo-dye technique. Arteries (a) and arterial capillaries (c) are *blackened*. There is no clear corticomedullary line (CM). Typical strongly reactive area in medulla (M) is seen in the *lower part* of photograph. A vein (v) with unreactive endothelium is at *right*. Cortex (C); oval cell (o); reticular cell (r).

FIGURE 4.—AKR thymus—alkaline phosphatase, glycerophosphate-cobalt sulfide technique. *Same thymus* as figure 3. A group of reticular cells are around *end of arrow*.

FIGURE 5.—C57BL/6 thymus—acid phosphatase, glycerophosphate-lead sulfide technique. Reactive cells are mostly in the medulla (M). Corticomedullary line (CM) is indistinct; cortex (C).

FIGURE 6.—AKR thymus—acid phosphatase, glycerophosphate-lead sulfide technique. Reactive cells are mostly in the cortex (C). Corticomedullary (CM) zone is sharply delimited; medulla (M).

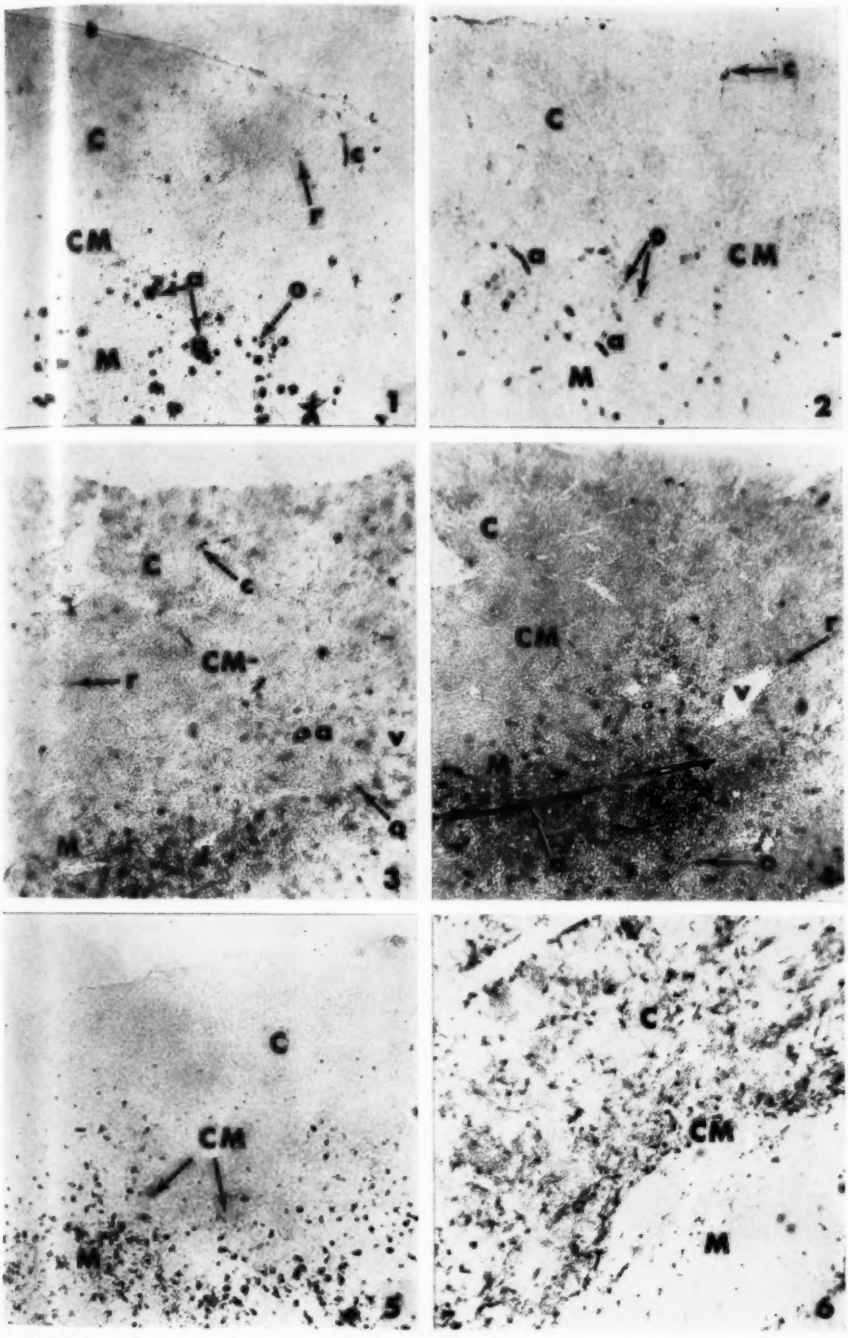
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PLATE 53

Thymuses of 4- to 5-week-old mice are shown in figures 7, 8, and 9. Alkaline phosphatase. The reactive medullary oval cell (o), glycerophosphate-cobalt sulfide method. Unfixed, frozen sections cut in cryostat. Green B58 filter

FIGURE 7.—C57BL/6 thymus. Nuclei of oval cells stained in safranin. Granular nature of reactive cytoplasm shows at *outer edge* of *upper cell*. A somewhat flattened nucleus (with nucleolus) *toward arrow* and cytoplasmic rim at *left* of nucleus is seen in lower cell, 5 μ. × 1433

FIGURE 8.—AKR thymus. Oval cell with no nuclear stain shows nucleus with irregular outline, nucleolus, and cytoplasmic rim, 10 μ. × 1,433

FIGURE 9.—C57BL/6 thymus. Oval cell, with much flattened nucleus, has become round. Few black granules can be seen in small cell *above*. No nuclear stain, 10 μ. × 1,100

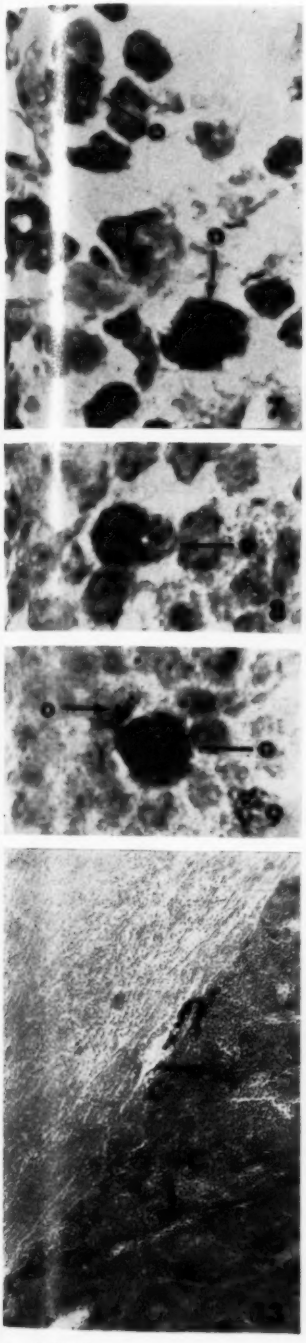
Thymuses of AKR retired breeders are shown in figures 10 to 13. Alkaline phosphatase, glycerophosphate-cobalt sulfide method, except where noted. Unfixed, frozen sections cut in cryostat, 10 μ. Green B58 filter. × 72, except where noted.

FIGURE 10.—AKR thymus of male—azo-dye technique. Thymus is of nearly normal size, both lobes about equal. One lobe is more reactive than the other; cortex (C); medulla (M). × 60

FIGURE 11.—AKR thymus of male. Involuted thymus. One lobe is much more reactive than the other. At *left* in strongly reactive lobe is section of a cyst lined by negative epithelium. With acid phosphatase technique the more reactive lobe has fewer positive cells and these are mostly in the medulla; less reactive lobe shows characteristic AKR picture, a more reactive cortex. In May-Grünwald-Giemsa preparation, cells in both lobes are of large lymphocytic type; mitoses are present in both lobes. Cortex (C); medulla (M); mediastinal connective tissue (met).

FIGURE 12.—AKR thymus of female. Large lobe, *upper part* of photograph, was judged 3× normal size. Cortical cells are intensely colored; remnants of pale medulla not included in photograph. In smaller lobe, clusters of more reactive cells are seen among less reactive cortical cells. With the acid phosphatase technique, large lobe possesses scattered positive cells; typical AKR cortex with many browned cells distinguishes smaller lobe. In May-Grünwald-Giemsa preparation, larger lobe is composed of large lymphocytes in mosaic pattern with basophilic cytoplasm, with many mitoses. Smaller lobe is also characterized by larger cells, more loosely arranged, some mitoses; cortex (C); medulla (M).

FIGURE 13.—AKR thymus of female. Large lobe, *upper part* of photograph, was judged 3× normal size. Cells are negative; invasion of capsule and interlobar connective tissue is seen. Smaller lobe is reactive. With acid phosphatase technique, larger lobe had numerous blackened cells; smaller lobe, a highly reactive cortex with negative nodules. In May-Grünwald-Giemsa preparation, large lobe is composed of large lymphocytes with many mitoses and numerous macrophages. In smaller lobe, cells vary from small to large lymphocytes depending on the section; cortex (C).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Distribution of Cancer Mortality Among Ethnic Subgroups of the White Population of New York City, 1953–58 [1, 2]

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SUMMARY

Data on 84,341 deaths of white residents in New York City occurring in the years 1953–58 and attributed to malignant neoplasm were examined with respect to nativity status and religion. Comparison was with estimates of the religious distribution of the population of the city and with a 2 percent sample of deaths from all causes in 1955. After age adjustment, total mortality rates from all neoplastic disorders were similar in the native-born and foreign-born populations, and differences between religious groups were not remarkable. Rates for the foreign-born and native-born for specific sites of malignant neoplasm showed relatively small differences. Data were not available to enable examination of cancer-mortality rates according to specific country of birth of the foreign-born. Most striking were the differences in rates of mortality from individual sites of neoplasm between the Jewish group and the Catholic and Protestant groups. Among males, cancers of the buccal cavity and pharynx, esophagus, gallbladder, larynx, lung, prostate, skin (other than melanoma), and other male genitalia were appreciably less common in the Jewish group than in either of the other religious groups. In females, cancer of the cervix uteri showed the same pattern. Certain sites exhibited higher rates in the Jewish group. These included cancers of the large intestine, kidney, brain, and thyroid, and melanoma, reticulum-cell sarcoma, lymphosarcoma, Hodgkin's disease, other lymphoma, and leukemia. Cancer of the male breast and, among females, cancers of the liver and pancreas, were also more common among the Jewish group. With some minor modifications, these patterns according to religion were seen in both the native-born and foreign-born populations.—J. Nat. Cancer Inst. 26: 405—417 1961.

COMPARISONS BETWEEN the white and Negro populations of the United States with respect to cancer frequency have been useful in epidemiologic investigations of neoplasms of such sites as lung, stomach,

[1] Received for publication August 11, 1960.

[2] This study was aided by Epidemiology Training Grant 2G-7 from the National Institutes of Health, Public Health Service.

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[4] The study was based on punch cards made from death certificates by the Department of Health of New York City. I am indebted to the Board of Health for permission to review death certificates and to Mr. Carl Erhardt and Mr. Louis Weiner of the Bureau of Records and Statistics of the Department of Health for making the data available.

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penis, testis, uterus, skin, and the hematopoietic system. Marked differences between the two racial groups in the frequencies of cancer of these sites have been demonstrated.

There are far fewer studies of the frequency of cancer among ethnic subgroups of the white population, primarily because routinely published statistical data do not identify such subgroups. Examining mortality data for Massachusetts, Lombard and Doering (*1*) found that the foreign-born in Massachusetts had a high age-adjusted death rate from cancer. They compared the rates of the native-born and foreign-born with social classes I and V in England,[5] respectively, and demonstrated a sufficiently close correlation to suggest to them that economic or social conditions were factors in the causation of cancer. They were also able to demonstrate variation in frequency of cancer of different sites among subgroups of the foreign-born population.

Sorsby (*2*) compared the Jewish and non-Jewish populations of London, Amsterdam, Vienna, Budapest, Warsaw, Lodz, and Leningrad. In London the rates used were proportional mortality rates and in the other cities either crude mortality rates or mortality rates specific for the cancer age (35 years and over in some cities and 40 years and over in others). On these data Sorsby commented that the total mortality from cancer among the Jews of any city followed closely the mortality among their fellow citizens and that on the whole there was greater similarity of cancer mortality between Jews and non-Jews in the same city than between Jews in different cities. However, with respect to specific sites of neoplasms, Jews appeared to have higher rates of cancer of the gastrointestinal tract and ovaries, and a lower mortality from cancer of the buccal cavity, uterus, and penis. Dorn (*3*) confirmed the low incidence of cancer of the cervix in Jewish women. MacMahon and Koller (*4*) demonstrated a high mortality from leukemia among the Jewish population of Brooklyn, New York.

In a systematic study of mortality from all the common sites of cancer in New York City in 1955, MacMahon (*5*) found that mortality rates for cancer of the stomach and for leukemia were about 50 percent higher in foreign-born than native-born. The death rate from cancer of the breast was 30 percent higher in the native-born than the foreign-born. Striking differences were also found between mortality rates in the 3 major religious groups, particularly between the Jewish and the other 2 groups. Cancers of the upper respiratory tract, uterine cervix, esophagus, liver, and lung were less common in Jews than in Catholics or Protestants. On the other hand, mortality from melanomas, gliomas, lymphomas, and neoplasms of the kidney, large intestine, and pancreas were substantially higher for the Jewish group than either of the other 2 groups.

The present paper reports an extension of the New York City study to include 6 years of mortality data rather than the 1 year originally examined (*5*). The purpose is to determine if the differences noted are

[5] Social class is determined by the occupation of the male. Professional people are social class I and unskilled workers social class V.

still evident in a larger sample of deaths and to examine sites of cancer for which numbers were inadequate in the earlier study.

MATERIAL

There were 92,562 deaths of New York City residents between 1953 and 1958 which were attributed to neoplastic disease (International List Numbers, 140–204). Of these 84,748 were classified as white, and information was available for tabulation on 84,341.[6] The numbers of deaths attributed to each of the 52 sites or site groups examined are shown in table 1.

Estimates of the age and sex distribution of the white population in 1955 were derived by linear interpolation between the censuses of 1950 (*6*) and 1957 (*7*). Data on nativity were not collected during the census of 1957. The distribution of the population according to native-born or foreign-born status was therefore estimated by application of the percentage distribution by nativity in 1950 in each sex and 5-year age group to the estimated population in 1955 in the same sex and age group.

NATIVITY

Among 84,341 deaths of white residents were 37,639 native-born, 46,418 foreign-born, and 284 of unknown nativity. The estimated native-born and foreign-born populations were 5,042,950 and 1,775,840, respectively, with average annual crude death rates from all sites of cancer of 124.4 per 100,000 in the native-born and 435.6 per 100,000 in the foreign-born. The discrepancy is entirely accounted for by the difference in age composition of the 2 populations. Standardization of the 2 rates to the age distribution of the white population of the city in 1950 results in average annual rates of 185.9 and 183.8 per 100,000 in the native-born and foreign-born, respectively. Age-adjusted rates for males and females in the native-born and foreign-born population for each site of cancer are shown in table 1. The age adjustment was by the direct method to the distribution of the white population in New York City in 1950 in 10-year age groups.

Only a few differences between the rates for native-born and foreign-born are large enough to warrant comment. Rates for the foreign-born were higher than those for the native-born for neoplasms of the esophagus, stomach, liver (primary), pancreas, and thyroid, and for multiple myeloma, and lymphatic, myeloid, and acute leukemia.

Rates for the native-born were higher than those for the foreign-born in both sexes for neoplasms of the lung (primary) and bladder, and for

[6] From the deck of IBM cards for the year 1954, 407 cards were missing. Since no irregularities in the distribution of the 1954 data by diagnosis, age, sex, race, nativity, or religion that might have been attributed to this deficit could be detected, the loss was ignored.

TABLE 1.—Average annual death rates per 100,000* from certain sites of cancer, New York City, resident white population, 1953–58

Site	Number of deaths	Average annual death rate per 100,000								
		All nativities			Native-born			Foreign-born		
		Male	Female	Total	Male	Female	Total	Male	Female	Total
All neoplasms (140–204)	84, 341	204. 5	151. 2	182. 4	212. 2	168. 2	185. 9	200. 8	166. 6	183. 8
Buccal cavity and pharynx (140–148)	1, 770	6. 5	1. 3	3. 8	7. 7	1. 5	4. 2	5. 7	1. 4	3. 5
Lip (140)	(33)†	(0. 1)	(0. 0)	(0. 1)	(0. 2)	(0. 0)	(0. 1)	(0. 1)	(0. 0)	(0. 1)
Tongue (141)	613	2. 3	0. 4	1. 3	2. 6	0. 5	1. 4	2. 0	0. 4	1. 2
Pharynx (145–148)	593	2. 2	0. 5	1. 3	2. 7	0. 5	1. 4	1. 9	0. 5	1. 2
Other (142–144)	531	1. 9	0. 4	1. 1	2. 3	0. 5	1. 2	1. 6	0. 4	1. 0
Digestive organs (150–159)	34, 219	85. 5	61. 2	72. 7	80. 2	57. 2	67. 0	86. 0	66. 2	76. 2
Esophagus (150)	1, 794	6. 2	1. 6	3. 8	5. 6	1. 4	3. 2	6. 6	1. 8	4. 1
Stomach (151)	8, 566	23. 2	13. 5	18. 1	19. 1	10. 3	14. 1	25. 5	16. 9	21. 0
Small intestine (152)	206	0. 4	0. 4	0. 4	0. 5	0. 4	0. 4	0. 4	0. 5	0. 4
Large intestine except rectum (153)	10, 045	22. 0	20. 7	21. 4	22. 0	21. 8	21. 7	21. 7	20. 1	20. 9
Rectum (154)	5, 160	13. 8	8. 5	11. 0	14. 5	9. 1	11. 4	13. 0	8. 4	10. 7
Liver, primary (155.0)	1, 354	2. 2	3. 5	2. 9	1. 7	2. 5	2. 2	2. 3	4. 0	3. 3
Gallbladder (155.1)	489	1. 0	1. 1	1. 0	1. 0	0. 9	1. 0	0. 9	1. 2	1. 0
Liver, secondary (156)	1, 822	4. 3	3. 5	3. 9	4. 3	3. 3	3. 7	4. 2	3. 8	4. 0
Pancreas (157)	4, 029	10. 6	6. 8	8. 6	9. 9	5. 8	7. 6	10. 7	7. 4	9. 0
Other (158–159)	754	1. 8	1. 5	1. 6	1. 6	1. 6	1. 6	0. 6	1. 9	1. 7
Respiratory system (160–164)	12, 504	47. 5	8. 2	26. 2	55. 0	7. 9	28. 8	41. 7	8. 4	25. 0
Larynx (161)	986	4. 1	0. 3	2. 1	5. 3	0. 4	2. 6	3. 3	0. 4	1. 8
Trachea (162.0)	216	0. 8	0. 2	0. 5	0. 8	0. 2	0. 5	0. 7	0. 2	0. 4
Lung and bronchus, primary (162.1)	4, 157	15. 8	2. 0	8. 6	19. 3	2. 0	9. 7	13. 3	1. 9	7. 6
Lung, secondary (163)	7, 093	26. 0	5. 4	14. 5	28. 6	5. 0	15. 4	23. 6	5. 5	14. 6
Other parts of (160–164)	52	0. 8	0. 3	0. 6	1. 0	0. 3	0. 6	0. 8	0. 4	0. 6
Breast (170)	8, 017	0. 4	33. 8	17. 9	0. 4	40. 4	22. 2	0. 4	28. 8	14. 5
Genital organs (171–179)	9, 139	14. 2	25. 6	19. 8	16. 7	28. 0	22. 1	12. 8	24. 9	18. 8
Uterus (171–174)	3, 215	—	13. 6	—	—	14. 8	—	—	13. 9	—
Cervix (171)	1, 704	—	7. 4	—	—	7. 5	—	—	8. 3	—
Corpus (172)	926	—	3. 8	—	—	4. 5	—	—	3. 3	—
Ovary (175.0)	2, 550	—	11. 0	—	—	12. 2	—	—	10. 0	—
Other female genital (176)	216	—	0. 8	—	—	0. 8	—	—	0. 9	—
Prostate (177)	2, 888	13. 0	—	—	15. 3	—	—	11. 7	—	—
Testes (178)	168	0. 9	—	—	1. 0	—	—	(0. 8)	—	—
Other male genital (179)	75	0. 3	—	—	(0. 4)	—	—	(0. 3)	—	—

Urinary organs (180–181)	4, 151	13. 0	5. 1	8. 8	14. 0	6. 0	9. 4	12. 3	4. 7	8. 5
Kidney (180)	1, 566	4. 6	2. 3	3. 4	4. 5	2. 3	3. 3	4. 7	2. 5	3. 6
Bladder (181.0)	2, 545	8. 3	2. 7	5. 3	9. 4	3. 6	6. 0	7. 6	2. 1	4. 8
Other and unspecified sites (165, 190–199)	7, 633	19. 1	14. 8	16. 9	20. 1	14. 6	17. 1	19. 3	15. 8	17. 6
Melanoma (190)	511	1. 3	1. 1	1. 2	1. 6	1. 2	1. 4	1. 0	0. 8	0. 9
Skin, other (191)	416	1. 2	0. 6	0. 9	1. 3	0. 7	0. 9	1. 2	0. 7	0. 9
Eye (192)	93	0. 2	0. 2	0. 2	(0. 2)	(0. 2)	(0. 2)	(0. 2)	(0. 2)	(0. 2)
Brain (193.0)	1, 036	2. 9	1. 9	2. 4	3. 0	1. 9	2. 4	3. 0	1. 9	2. 4
Nervous system, other (193.1–193.2)	816	2. 2	1. 6	1. 9	2. 1	1. 5	1. 8	2. 9	1. 9	2. 4
Thyroid (194)	417	0. 6	1. 1	0. 9	0. 5	0. 9	0. 7	0. 8	1. 3	1. 1
Adrenal (195.0)	83	0. 3	0. 1	0. 2	(0. 3)	(0. 1)	(0. 2)	(0. 2)	(0. 2)	(0. 2)
Endocrine glands, other (195.1–195.8)	61	0. 2	0. 1	0. 1	(0. 2)	(0. 1)	(0. 2)	(0. 1)	(0. 5)	(0. 3)
Bone (196)	578	1. 5	1. 1	1. 3	1. 5	1. 1	1. 3	1. 6	1. 1	1. 4
Other and unspecified (165, 198–199)	3, 278	7. 8	6. 4	7. 0	8. 4	6. 4	7. 3	7. 4	6. 3	6. 9
Other specified (197)	304	0. 8	0. 6	0. 7	0. 8	0. 5	0. 6	0. 9	0. 7	0. 8
Lymphosarcoma and other lymphatic and hematopoietic (200–203)	3, 710	9. 9	7. 0	8. 4	9. 7	7. 1	8. 3	11. 2	6. 3	9. 2
Reticulum-cell sarcoma (200.0)	504	1. 4	1. 0	1. 2	1. 5	1. 0	1. 2	1. 2	0. 9	1. 0
Lymphosarcoma (200.1)	1, 163	3. 0	2. 2	2. 6	3. 0	2. 2	2. 6	3. 9	2. 3	3. 2
Other primary lymphoid (200.2)	190	0. 5	0. 3	0. 4	0. 5	0. 4	0. 4	0. 6	0. 3	0. 5
Hodgkin's disease (201)	989	2. 8	2. 0	2. 4	2. 8	2. 1	2. 4	2. 7	2. 1	2. 4
Lymphoma, other (202)	173	0. 5	0. 3	0. 4	0. 5	0. 3	0. 4	0. 4	0. 3	0. 4
Multiple myeloma (203)	691	1. 7	1. 3	1. 5	1. 4	1. 1	1. 2	2. 2	1. 4	1. 8
Leukemia and aleukemia (204)	3, 197	8. 4	6. 1	7. 2	8. 2	5. 7	6. 8	10. 4	10. 8	10. 6
Lymphatic (204.0)	922	2. 6	1. 5	2. 0	2. 4	1. 2	1. 7	3. 7	2. 4	3. 0
Myeloid (204.1)	1, 165	2. 9	2. 4	2. 6	3. 0	2. 3	2. 6	2. 9	3. 6	3. 3
Monocytic (204.2)	116	0. 3	0. 2	0. 3	0. 3	0. 2	0. 3	0. 2	0. 2	0. 2
Acute (204.3)	682	1. 7	1. 5	1. 6	1. 8	1. 4	1. 6	2. 4	4. 0	3. 2
Other and unspecified (204.4)	312	0. 8	0. 6	0. 7	0. 7	0. 6	0. 7	1. 2	0. 6	0. 9

*Age-standardized to the New York City population, 1950.

†Parentheses indicate numbers based on fewer than 50 deaths in the 6 years.

melanoma; rates for native-born females were higher for neoplasms of the breast, corpus of the uterus and ovary, and for males for neoplasms of the larynx, prostate, buccal cavity, and pharynx. Higher rates of neoplasms of the liver and bone noted by MacMahon in the native-born are not confirmed in the larger sample. The higher rate of skin cancer noted in the earlier data is now seen to be due to the component melanoma.

The fact that age distibutions of the appropriate subgroups of the population are not known precludes the possibility of examining cancer mortality rates according to the specific country of birth of the foreign-born. Comparison of the crude and age-adjusted rates for the foreign-born as a group points up the fact that without age adjustment nativity comparisons can be extremely misleading.

RELIGION

Denominators for the computation of rates in the 3 main religious groups of the city, Catholic, Jewish, and Protestant, were obtained from the sample survey undertaken by the Health Insurance Plan of Greater New York in 1952 (*8*). Data were presented from this survey on the estimated percentage of the population in each religious group in the age groups 0 to 14, 15 to 44, 45 to 64, and 65 and over. These groups are too broad for effective standardization; however, the distributions in the groups 45 to 64 and 65 and over are very similar. The examination has therefore been restricted to the population aged 45 and over, without further age adjustment within that group, on the assumption that variation in the religious distribution of the population by age is inconsequential after age 45.

Numerators are derived from the item "religious affiliation of cemetery of burial" that has been routinely coded and punched into the death card by the Department of Health. Comparing religions as stated on hospital records with religion as inferred from the above item on the death cards in a series of patients dying of leukemia, MacMahon and Koller (*4*) noted that persons buried in Catholic and Jewish cemeteries were almost exclusively Catholic or Jewish, respectively, while of those buried in Protestant and nonsectarian cemeteries and those cremated, 63 percent were Protestant, 22 percent Catholic, 8 percent Jewish, and 7 percent of other religious affiliation. Therefore, in the estimation of the religious distribution of the cancer deaths in the present study, all persons buried in Catholic or Jewish cemeteries have been assumed to have been Catholic or Jewish, respectively; those buried in cemeteries other than these have been distributed in accordance with this pattern.

Two assumptions that may influence the rates derived have been made: 1) Age variation in the religious distribution can safely be discounted after age 45; 2) the different methods used for deriving religion in the numerator and the denominator yield sufficiently comparable results.

These two assumptions appear justified not only on the basis of the evidence already presented, but also by the fact that MacMahon (*5*) examined the data for 1955 both by the method described here and by another method which did not depend on either of these assumptions, but yielded proportional mortality estimates rather than mortality rates. Results, as far as comparison of mortality patterns in the three religious groups is concerned, were very similar by the two methods. Since rates are much more readily interpreted than proportional mortalities, only the one method has been used in this examination.

The data are given in table 2. The total death rates from neoplastic disease do not differ remarkably in the three religious groups. In spite of the well-known infrequency of cervical cancer among Jewish women, this group has a total death rate from cancer about 15 percent higher than either of the other groups of women. Among males the total rate is a little higher for Protestants than for the other two groups.

However, much greater variation is seen in the rates for individual sites or groups of sites. The most striking differences are seen in comparison of the Jewish with the other two religious groups. Certain sites were less common in the Jewish group: among males, cancers of the buccal cavity and pharynx, esophagus, gallbladder, larynx, lung and bronchus, prostate, skin (other than melanoma), and other male genitalia; and among females, cancer of the cervix uteri. Other sites were more common among Jews than either Catholics or Protestants; sites in which this was true in both sexes include large intestine, kidney, brain and thyroid, and melanoma, reticulum-cell sarcoma, lymphosarcoma, Hodgkin's disease, other lymphoma, multiple myeloma, and leukemia; among males, cancer of the breast, and among females, cancer of the stomach, liver (primary), pancreas, and lung (secondary).

Differences between Catholics and Protestants are small and hardly worthy of comment, though there is an over-all impression that, in a large number of different sites, rates for Protestants are slightly higher than those for Catholics.

RELIGION AND NATIVITY

Since the main differences demonstrated in table 2 are between the Jewish population and the Catholic and Protestant groups, these latter have been combined in table 3 to show the ratios of the frequencies of individual sites to their frequencies in non-Jewish separately in the native-born and foreign-born populations.

Since population data are not available by religion and nativity simultaneously, it has been necessary to use the proportional mortality technique already mentioned. A 2 percent systematic sample of all deaths of New York City residents in 1955 was used to estimate the distribution of all deaths in the period 1953–58 by religion, nativity, and age. Age-standardized proportions of all deaths attributed to a particular neoplasm

TABLE 2.—Estimated average annual death rates per 100,000 from certain sites of cancer in 3 religious groups, New York City, resident white population age 45 years or over, 1953–58

Site	Male			Female			Both sexes		
	Catholic	Jewish	Protestant	Catholic	Jewish	Protestant	Catholic	Jewish	Protestant
All neoplasms (140–204)	578.1	572.3	623.0	430.3	516.0	450.7	504.4	543.5	533.9
Buccal cavity and pharynx (140–148)	25.0	7.7	21.3	3.9	3.4	4.3	14.0	5.5	12.5
Lip (140)	(0.5)*	(0.1)	(0.5)	(0.1)	(0.0)	(0.0)	(0.3)	(0.1)	(0.2)
Tongue (141)	8.7	2.5	7.8	1.2	1.5	1.3	4.8	2.0	4.4
Pharynx (145–148)	8.4	2.6	7.9	1.3	0.8	1.5	4.7	1.7	4.6
Other (142–144)	7.3	2.5	5.1	1.3	1.0	1.5	4.2	1.7	3.2
Digestive organs (150–159)	247.8	269.7	254.9	180.2	219.0	178.4	213.8	243.4	215.2
Esophagus (150)	21.9	12.3	20.5	3.9	6.0	5.4	12.6	9.0	12.6
Stomach (151)	64.8	74.1	72.7	39.9	50.1	40.7	51.9	61.6	56.1
Small intestine (152)	1.3	1.1	1.8	1.3	1.4	1.3	1.3	1.3	1.6
Large intestine except rectum (153)	59.1	81.4	59.8	59.9	74.5	60.2	59.5	77.9	60.0
Rectum (154)	43.2	39.8	40.5	27.2	26.3	25.0	34.9	32.8	32.4
Liver, primary (155.0)	6.6	6.6	6.2	10.4	14.1	8.8	8.6	10.5	7.6
Gallbladder (155.1)	3.3	2.2	3.9	3.3	4.2	2.2	3.4	3.2	3.0
Liver, secondary (156)	13.9	11.0	13.6	10.1	12.5	10.5	11.9	11.8	12.0
Pancreas (157)	28.6	35.8	31.0	19.7	25.2	19.9	24.0	30.3	25.3
Other (158–159)	5.1	5.5	5.0	4.4	4.6	4.4	4.7	5.0	4.7
Respiratory system (160–164)	151.1	113.7	165.6	20.1	31.0	21.9	83.2	70.8	91.0
Larynx (161)	14.9	6.2	15.7	1.1	0.7	0.8	7.8	3.3	8.0
Trachea (162.0)	2.3	2.1	2.2	0.5	0.8	0.6	1.4	1.4	1.4
Lung and bronchus, primary (162.1)	50.0	34.0	60.0	4.8	7.0	5.0	26.6	20.0	31.4
Lung, secondary (163)	81.7	69.8	85.2	12.7	21.6	14.5	45.9	44.8	48.5
Other parts (160–164)	2.2	1.6	2.5	1.0	1.0	1.0	1.6	1.3	1.7
Breast (170)	1.0	1.7	0.9	84.0	95.6	90.4	44.0	50.3	47.4
Genital organs (171–179)	41.8	34.5	53.6	68.5	63.2	78.6	55.6	49.4	66.8
Uterus (171–174)	—	—	—	39.9	27.2	46.1	—	—	—
Cervix (171)	—	—	—	22.0	9.0	24.3	—	—	—
Corpus (172)	—	—	—	10.5	12.6	12.2	—	—	—
Ovary (175.0)	—	—	—	25.4	33.5	28.9	—	—	—
Other female genital (176)	—	—	—	2.9	2.0	3.3	—	—	—
Prostate (177)	40.0	33.0	51.3	—	—	—	—	—	—
Testes (178)	0.6	1.0	1.0	—	—	—	—	—	—
Other male genital (179)	1.1	0.5	1.3	—	—	—	—	—	—

Urinary organs (180–181)	34.8	42.4	41.3	15.3	15.9	17.1	24.7	28.7	28.8
Kidney (180)	9.7	17.8	12.6	5.8	8.5	5.6	7.7	13.0	8.9
Bladder (181.0)	24.8	24.5	28.5	9.2	7.3	11.0	16.7	15.6	19.4
Other and unspecified sites (165, 190–199)	47.4	50.4	47.5	35.0	46.1	35.0	41.0	48.1	41.0
Melanoma (190)	2.3	3.4	2.7	1.9	2.8	2.1	2.1	3.1	2.4
Skin, other (191)	4.2	2.1	3.4	2.4	1.4	1.6	3.2	1.8	2.5
Eye (192)	(0.4)	(0.5)	(0.6)	(0.4)	(0.9)	(0.5)	0.4	0.7	0.6
Brain (193.0)	5.1	8.2	6.1	3.2	5.6	3.2	4.1	6.9	4.6
Nervous system, other (193.1–193.2)	4.8	5.6	4.9	2.7	4.1	3.0	3.7	4.8	3.9
Thyroid (194)	1.7	2.4	1.4	3.0	4.5	3.0	2.4	3.5	2.3
Adrenal (195.0)	(0.6)	(0.5)	(0.4)	(0.3)	(0.3)	(0.2)	0.4	0.4	0.3
Endocrine glands, other (195.1–195.8)	(0.4)	(0.2)	(0.4)	(0.1)	(0.3)	(0.2)	(0.2)	(0.3)	(0.3)
Bone (196)	4.2	3.4	3.1	2.4	3.0	2.9	3.3	3.2	3.0
Other and unspecified (165, 198–199)	22.3	21.7	22.7	17.4	21.3	17.0	19.8	21.5	19.8
Other specified (197)	1.5	2.3	1.7	1.1	1.8	1.2	1.3	2.1	1.4
Lymphosarcoma, other lymphatic and hematopoietic (200–203)	14.3	30.2	21.1	13.1	23.6	13.9	15.6	26.7	17.4
Reticulum-cell sarcoma (200.0)	2.0	4.1	3.1	1.9	3.4	1.7	2.0	3.8	2.4
Lymphosarcoma (200.1)	6.0	10.6	6.3	4.2	8.4	4.5	5.1	9.5	5.4
Other primary lymphoid (200.2)	1.0	1.2	1.2	0.9	0.8	1.2	1.0	1.0	1.2
Hodgkin's disease (201)	4.2	5.6	4.8	2.2	4.7	3.0	3.2	5.2	3.8
Lymphoma, other (202)	1.0	1.6	0.6	0.6	1.1	0.6	0.8	1.4	0.6
Multiple myeloma (203)	4.1	6.9	5.2	3.3	5.1	3.0	3.7	6.0	4.1
Leukemia and aleukemia (204)	15.1	22.7	16.7	10.2	18.4	11.2	12.5	20.5	13.8
Lymphatic (204.0)	5.8	8.4	6.0	3.1	5.8	2.9	4.4	7.0	4.4
Myeloid (204.1)	5.1	8.1	5.9	3.9	7.2	5.0	4.5	7.6	5.5
Monocytic (204.2)	0.7	0.8	0.8	(0.4)	(0.6)	(0.3)	0.5	0.7	0.6
Acute (204.3)	1.9	3.3	2.2	1.6	3.1	1.8	1.7	3.2	2.0
Other and unspecified (204.4)	1.5	2.2	1.6	1.2	1.7	1.2	1.4	1.9	1.4

*Numbers in parentheses are based on fewer than 50 deaths in the 6 years.

were then calculated separately for the native-born and foreign-born and by religion. The ratios between these proportions in the Jewish and the non-Jewish are the data shown in table 3. As already noted, for all nativity groups combined, this method gives results similar to the direct calculation of mortality rates when the necessary population data are available.

In general the relationships are similar for the two nativity groups, though the higher frequencies of cancers of the breast, ovary, and testis, and of melanoma, glioma, and lymphoma are less striking in the foreign-born than in the native-born. The relative deficiency of upper respiratory cancer in Jews is about equally evident in both nativity groups. The deficiency of cervical cancer in Jewish women is most marked in the foreign-born.

TABLE 3.—Ratio of frequency with which specific sites of cancer are recorded as cause of death in Jewish population compared to frequency of 1.0 in non-Jewish population, New York City, 1953–58

Site	Both sexes		
	Native-born	Foreign-born	All nativities
All neoplasms (140–204)	1.3	1.0	1.2
Buccal cavity and pharynx (140–148)	0.4	0.4	0.4
Lip (140)	0.0	0.2	0.2
Tongue (141)	0.5	0.4	0.4
Pharynx (145–148)	0.4	0.3	0.3
Other (142–144)	0.5	0.4	0.5
Digestive organs (150–159)	1.2	1.1	1.2
Esophagus (150)	0.2	0.8	0.7
Stomach (151)	1.2	1.0	1.2
Small intestine (152)	0.9	0.9	0.9
Large intestine except rectum (153)	1.4	1.4	1.4
Rectum (154)	1.1	0.9	1.0
Liver, primary (155.0)	1.1	1.2	1.3
Gallbladder (155.1)	1.3	0.9	1.0
Liver, secondary (156)	1.0	0.9	1.0
Pancreas (157)	1.4	1.1	1.3
Other (158–159)	1.4	1.0	1.2
Respiratory system (160–164)	0.7	0.9	0.8
Larynx (161)	0.4	0.4	0.4
Trachea (162.0)	1.5	1.0	1.1
Lung and bronchus, primary (162.1)	0.6	0.8	0.7
Lung, secondary (163)	0.8	1.0	1.0
Other parts (160–164)	1.5	0.6	0.8
Breast (170)	2.0	1.2	1.4
Genital organs (171–179)	1.3	0.8	0.9
Uterus (171–174)	0.9	0.6	0.7
Cervix (171)	0.6	0.3	0.4
Corpus (172)	1.4	1.2	1.2
Ovary (175.0)	2.0	1.4	1.6
Other female genital (176)	0.7	0.6	0.6
Prostate (177)	0.8	0.7	0.7
Testes (178)	2.4	1.3	2.0
Other male genital (179)	0.8	0.3	0.4
Urinary organs (180–181)	1.2	1.1	1.1
Kidney (180)	1.8	1.5	1.7
Bladder (181.0)	0.9	0.9	0.9

TABLE 3.—Ratio of frequency with which specific sites of cancer are recorded as cause of death in Jewish population compared to frequency of 1.0 in non-Jewish population, New York City, 1953–58—Continued

Site	Both sexes		
	Native-born	Foreign-born	All nativities
Other and unspecified sites (165, 190–199)	1.6	1.1	1.3
Melanoma (190)	2.6	1.4	1.9
Skin, other (191)	0.7	0.6	0.6
Eye (192)	1.6	1.8	1.7
Brain (193.0)	2.0	1.5	1.8
Nervous system, other (193.1–193.2)	1.7	1.2	1.6
Thyroid (194)	1.7	1.3	1.6
Adrenal (195.0)	1.8	0.8	1.3
Bone (196)	1.6	0.9	1.2
Other and unspecified (165, 198–199)	1.1	1.1	1.1
Other specified (197)	2.1	1.7	1.9
Lymphosarcoma and other lymphatic and hematopoietic (200–203)	2.5	1.5	1.9
Reticulum-cell sarcoma (200.0)	3.0	1.5	2.1
Lymphosarcoma (200.1)	2.5	1.7	1.9
Other primary lymphoid (200.2)	1.1	0.9	1.0
Hodgkin's disease (201)	2.9	1.4	2.1
Lymphoma and other (202)	3.0	1.7	2.2
Multiple myeloma (203)	1.7	1.5	1.7
Leukemia and aleukemia (204)	2.1	1.5	1.9
Lymphatic (204.0)	1.3	1.5	1.6
Myeloid (204.1)	2.4	1.6	2.0
Monocytic (204.2)	1.9	1.3	1.6
Acute (204.3)	2.4	1.6	2.2
Other and unspecified (204.4)	2.1	1.2	1.6

DISCUSSION

The low frequency of cancer of the uterine cervix among Jewish women is of such epidemiologic interest that preoccupation with this neoplasm appears to have obscured many other equally marked and fascinating characteristics of the experience of Jewish persons with neoplastic disease. We may speculate that the range of explanation of this experience includes characteristics related to religious affiliation, *e.g.*, circumcision, as well as dietary, recreational, occupational, and socioeconomic characteristics related to a certain degree of homogeneity of ancestral and nativity background that is only accidentally associated with religion.

It does not seem profitable to speculate on specific explanations of patterns seen within any particular category of neoplasm except in the context of a complete review of ancillary knowledge of other epidemiologic characteristics of that category of neoplasm. It may be useful, however, to point to the value of knowledge of these patterns in such a context. One may question whether the hypothesis of relationship of cancer of the uterine cervix to lack of circumcision of the sexual partner would have been formulated without knowledge of the low frequency of this neoplasm in Jewish women. Whether or not this hypothesis is eventually substan-

tiated, the absence of other serious contenders has made it a useful focus for a great deal of profitable investigation.

To select, somewhat arbitrarily, two of the patterns noted in the present material as particularly worthy of further attention, one is drawn to the observations regarding cancer of the buccal cavity and pharynx and cancer of the large intestine. The first is of interest because of the extent of the differences noted (Jewish males have only one third the mortality rate of the other two groups) and because of the sex difference in the relationship (no appreciable difference between religious groups is evident in the data for females, who have very low mortality rates from this neoplasm). It would appear that the factors leading to a high frequency of this condition in males as compared to females are less frequent, or less strong, among Jewish as compared to non-Jewish males. A somewhat similar pattern is seen for neoplasms of the larynx and esophagus.

The second pattern, seen in cancers of the large intestine, is of interest not so much because of the extent of the differences noted (rates are only about 30% higher in Jews than in the other two groups), but because of the numerical importance of this particular site of neoplasm and because, with the exception of age, virtually no other important sources of variation in frequency of this neoplasm are known.

The general similarity of rates of individual neoplasms in Catholics and Protestants should not be interpreted as indicating that the ethnic groups contained within these religious categories do not have unusual cancer experience. While a majority of the Jewish population of New York City is of East European ancestral background, the Catholic and Protestant populations are more heterogeneous. The former includes as major components persons of Irish, Italian, and Puerto Rican background; the latter includes descendants of English, German, and other nationalities. An unusual cancer experience of any one of these groups could readily be hidden by dissimilar or contrary patterns exhibited by other nationality groups within the religious category. While the absence of variation in cancer rates according to religion may, then, be attributed to such considerations, the fact that major differences are found in the Jewish group indicates that this category of the religious classification, or at least a large section of the members of this category, has some homogeneity of experience that is meaningful in terms of cancer mortality.

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Studies of Some Antigens of the L Strain Mouse Fibroblast [1, 2, 3]

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SUMMARY

Agglutination, complement-fixation, and metabolic inhibition reactions were used to compare the cross-reactivity of the L strain mouse fibroblast with other cell strains grown *in vitro*. A high degree of cross-reactivity was observed between 2 lines of mouse fibroblasts (strains L and LLC-M_1) or between 2 lines of human epithelial cells (HeLa and Chang liver). A lower order of cross-reactivity was noted between the fibroblasts and epithelial cells. No antigenic differences could be detected between strain L and strain LLC-M_1 fibroblasts. Metabolic inhibition titers of heat-inactivated antiserums to fibroblasts strains L or LLC-M_1 were found to be low. With the addition of fresh guinea-pig serum, the inhibition titers increased fivefold. A serologic characterization was undertaken of the surface antigens of the strain L cells. Cells were treated with several enzymes or chemical agents known to have effects on different chemical groupings. It was found that treatment with sodium periodate or lipase abolished the agglutination of cells by antiserum prepared against whole, untreated cells. Trypsin, pancreatin, and collagenase, in low concentration, increased the titer. With higher concentrations or prolonged exposure to trypsin, there was a nonspecific aggregation of cells. Several chemical procedures were used to fractionate the strain L cells and prepare antigens of varying chemical composition. A water-methanol extract of the fibroblasts stimulated the production of agglutinating, complement-fixing, and cytotoxic antibodies reacting with whole cells. Antibodies to this antigen cross-reacted with strain LLC-M_1.—J. Nat. Cancer Inst. 26: 419–434, 1961.

MAMMALIAN CELLS grown in tissue culture have been reported to exhibit cross-reactions with antiserums to cells from other organs of the same species (*1–3*) as well as to cells of other species (*3, 4*). For these and other reasons, the usefulness of serologic methods for the characterization of cells grown *in vitro* has been questioned by some workers and has been considered to be of limited value by others.

[1] Received for publication August 15, 1960.

[2] Based in part on a thesis submitted as partial fulfillment of the requirements for the Ph. D. degree in Bacteriology, The University of Michigan, 1959.

[3] This work was supported by grant C-2539 from the National Cancer Institute, National Institutes of Health, Public Health Service.

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Despite the considerable amount of attention that has been directed to tissue-cell antigens as related to problems in tissue transplantation, the detection and recognition of tumor-specific antigens, the nature of iso-antigens, and similar problems, surprisingly little effort has been made to analyze the antigenic structure of cells grown in tissue culture by the classic methods that have proved so rewarding in the investigations of the antigenic structure of microorganisms. In particular there has been little concern with the relationship of the antigens studied and morphologic structures of the cell, though Latta (*5*) has used the technique of electron microscopy to characterize the action of antibody and complement on the cell membranes. Flax (*6*) found that antibody to ascites tumor cells caused the loss of cellular capacity to metabolize glucose with retention of capacity to utilize succinate.

Defendi and Colter (*4*) prepared antiserums against nucleoprotein fractions of several established cell lines and demonstrated cytotoxic effects that could not be correlated either to species or to tissue of origin. Coriell *et al.* (*3*) observed that many long-term tissue-culture cell lines tended to acquire common antigens or to lose specific ones. The common antigens frequently crossed species lines. Brand and Syverton (*7*), using a hemagglutination technique, reported that species of origin could readily be determined even after long periods in culture.

The present paper presents studies of the surface antigens of the L strain mouse fibroblast grown *in vitro*. This cell line, chosen because of its unique combination of properties, was isolated as a clone in 1948 (*8*). It grows readily in simple nutrient solutions and was shown to be readily adapted to serologic procedures that could be quantitated (*9*).

In the study reported here, the effects of enzymes and of chemical agents on the surface of the L strain cell have been correlated with the results of serologic studies. In addition, several antigenic components have been separated by chemical fractionation procedures and the reactivity of the antiserums to these fractions with whole cells has been studied. The specificity of the antigens of the L strain has been tested in cross-reactions with another mouse fibroblast strain and with two human epithelial cell lines.

MATERIALS AND METHODS

Cell strains.—The cell strains used in these experiments were the strain L clone 929 mouse fibroblast of Earle (*8*), the LLC-M_1 mouse fibroblast isolated by Hull (*10*), the HeLa strain of human cervical carcinoma isolated by Gey (*11*), and Chang's strain of human liver epithelial cells (*12*). Each of these cell lines had been maintained in this laboratory for at least 5 years at the time this study was made.

Culture media.—The nutrient solutions used were medium 199 of Morgan, Morton, and Parker (*13*) and Eagle's basal medium (*14, 15*). These media were modified as follows: For growth of strains L and LLC-M_1 in monolayers, medium 199 plus 5 percent whole horse serum was

initially used but was later replaced by 199 plus 0.5 percent peptone (Bacto-peptone, Difco Laboratories, Detroit, Mich.). As an alternative to the latter medium the following was also used: Eagle's synthetic mixture plus 0.05 percent yeast extract, 0.25 percent lactalbumin hydrolysate, and 0.5 percent peptone (YELP). In suspension cultures 0.12 percent methylcellulose (Methocel, 15 cps, reagent grade, supplied through the courtesy of Dow Chemical Company, Midland, Mich.) has been employed to replace horse serum, with the use of either YELP or 199-peptone medium. For the growth in monolayers of the two epithelial strains, HeLa and Chang liver, the medium used was Eagle's basal medium plus 0.05 percent yeast extract, 0.25 percent lactalbumin hydrolysate, and 20 percent whole human serum. Penicillin G and dihydrostreptomycin sulfate were added to bring the initial concentration to 100 units and 100 μg/ml, respectively.

Growth of tissue cells.—Tissue cells were grown as stationary cultures in Neutraglas bottles of 250 ml capacity. We harvested the cells by scraping them from the glass with a rubber policeman. For transfer purposes, the HeLa and Chang liver strains were treated with 0.25 percent trypsin (Trypsin, 1:250, Difco Laboratories, Detroit, Mich.), but the scraping procedure was applied when the cells were to be used for immunization or serologic tests (except the metabolic inhibition tests). Strains L and LLC-M_1 also were grown in agitated suspension cultures in Erlenmeyer flasks closed with rubber stoppers. Three sizes of flasks were used: 250 ml capacity, with 80 to 100 ml of medium; 500 ml capacity, with 100 to 150 ml medium; and 2000 ml capacity, with 1200 ml medium. The medium was agitated with a rotary shaker having a 1-inch radial stroke. The speed was adjusted to 100 rpm. Using this procedure, Kuchler and Merchant (*16*) found the cells in shaken cultures to grow in a homogenous suspension during the logarithmic phase. Cells in this phase of growth were used exclusively for the studies reported here.

Production of antiserums.—Cells were washed 3 times in Veronal-buffered saline at *p*H 7.0, and a total of 1×10^7 cells was inoculated subcutaneously into both hind footpads of rabbits. Five injections were given at 3- to 5-day intervals. Each of 30 rabbits was inoculated with either strain L, LLC-M_1, HeLa; or Chang liver cells. Bleedings were made 7 days after the last inoculation. The serum was collected, heat inactivated at 56° C for 30 minutes, stored at −20° C, and tested individually. For the production of antiserums to cell extracts, Freund's adjuvant (complete, Difco Laboratories, Detroit Mich.) was incorporated with a saline suspension of the lyophilized material and injected as described. One to 2 mg of lyophilized extract was used as the inoculum. At least 2 rabbits were inoculated with each extract.

Agglutination test.—Optimal conditions for agglutination tests with tissue cells were established and reported by Kite *et al.* (*9*). Serial dilutions of serums were made in 0.005 M Versene, a mixture of disodium and tetrasodium salts of ethylenediaminetetraacetic acid adjusted to give a final *p*H of 7.0 in 0.85 percent NaCl as a diluent.

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Complement-fixation test.—The 50 percent endpoint method of complement fixation was used as described by Kabat and Mayer (*17*). To 0.2 ml of each serum dilution, an equal volume of whole cells (5×10^5/ml) was added. The 50 percent unit of complement in 0.4 ml was added, the tests were incubated at 7° C for 18 hours, and 0.4 ml of sensitized sheep red blood cells was added. The tests were incubated at 37° C for 2 hours and 1.8 ml of cold Veronal-buffered saline was added. We determined the degree of hemolysis after centrifugation by reading the optical density of the supernatant fluid in a Coleman Nephelo-colorimeter with a 525 mμ filter.

Metabolic inhibition test.—In this laboratory Kuchler (*18*) developed a test that measures the ability of tissue cells to metabolize in the presence of antiserum. This test is a modification of that developed by Rightsell *et al.* (*19*) for virus assay. All cell and serum dilutions were made in growth medium adjusted to a *p*H of approximately 7.8. Twofold serial dilutions were prepared with each serum and 0.5 ml of each dilution was added to a cup or depression in a plastic panel tray. (Vinyl cup panels were obtained from Fabri-Kal Corp., Kalamazoo, Mich. The cup volume was 1.0 ml). The L and LLC-M_1 mouse fibroblasts were scraped from the surface of bottles with a rubber policeman. HeLa and Chang liver cells (human) were harvested with 0.02 percent Versene in balanced salt solution to obtain a suspension that could be counted accurately and would be uniformly dispersed for reaction with antiserum. A volume of 0.5 ml of cell suspension was added to each serum dilution. When the addition of complement was desired, 0.1 ml of guinea-pig serum, pooled and stored at −20° C, was added. The entire row of cups was sealed with a strip of cellophane tape,[5] shaken and incubated at 37° C. A final concentration of 20,000 cells per cup and a 1:10 final dilution of guinea-pig serum were used. Color changes of the medium due to metabolic activity were observed in 5 to 7 days. Microscopic observation was made by inversion of the plastic panel.

Enzymatic and chemical treatment of whole cells.—Whole cells were washed 3 times in Veronal-buffered saline, *p*H 7.0. The desired concentration of reagent (enzyme or chemical agent) was prepared in Veronal-buffered saline at *p*H 7.0, and 5 ml was added to the washed and packed cells. The cells were mixed well, allowed to stand at room temperature for 30 minutes with frequent mixing, washed twice with 0.005 M Versene in saline, and then used in agglutination tests.

Fractionation procedures performed on the strain L fibroblast.—Several established procedures were used in the fractionation of cells. These were selected to obtain fractions of several combinations as regards protein, carbohydrate, and lipide. The procedures are given in table 1. Cells were washed 3 times in Veronal-buffered saline, *p*H 7.0, and 2 to 4 g, wet weight, was used immediately for each fractionation procedure.

Methods of chemical analysis.—Whole cells were washed 3 times in

[5] Scotch brand cellophane tape #600, 2 inches wide, obtained from the Minnesota Mining and Manufacturing Co., St. Paul, Minn.

Veronal-buffered saline, *p*H 7.0, and chemical determinations were immediately performed on the wet-packed or resuspended cells. Nitrogen was determined by the Markham (*20*) modification of the micro-Kjeldahl determination, hexose by the anthrone method of Graff and Dreywood (*21, 22*) and hexosamine by the Blix modification (*23*) of the Elson and Morgan method (*24*). For the analysis of deoxyribonucleic acid (DNA)

TABLE 1.—Chemical extraction of strain L fibroblasts

Extract No.	Type of extract	Method of preparation
1	Aqueous	Cells + distilled H_2O. Let stand 2 to 8 hours at 7° C. Centrifuged at 3000 rpm for 15 minutes. Supernatant fluid dialyzed against distilled H_2O at 7° C and lyophilized.
2a	$(NH_4)_2SO_4$ precipitate	Cells + distilled H_2O. Sonic vibration for 3 minutes. The resulting suspension was precipitated with 20% saturated $(NH_4)_2SO_4$ at 7° C. Precipitate dialyzed against distilled H_2O at 7° C and lyophilized.
2b	"	Supernatant fluid from 2a precipitated with 30% saturated $(NH_4)_2SO_4$ at 7° C. Precipitate dialyzed against distilled H_2O at 7° C and lyophilized.
2c	"	Supernatant fluid from 2b precipitated with 40% saturated $(NH_4)_2SO_4$ at 7° C. Precipitate dialyzed against distilled H_2O at 7° C and lyophilized.
2d	"	Supernatant fluid from 2c precipitated with 50% saturated $(NH_4)_2SO_4$ at 7° C. Precipitate dialyzed against distilled H_2O at 7° C and lyophilized.
2e	"	Supernatant fluid from 2d dialyzed against distilled H_2O at 7° C and lyophilized.
3a	Ethanol precipitate of TCA soluble fraction	Cells + distilled H_2O. Either given no further treatment or disrupted by sonic vibration for 3 minutes or the Lourdes disintegrator for 3 minutes. Addition of equal volume of cold 0.5 M trichloracetic acid, allowed to stand at 7° C for 3 hours and centrifuged at 3000 rpm for 15 minutes. Supernatant fluid was saturated with NaCl + EtOH (2–3 vol.). Precipitate dialyzed against distilled H_2O at 7° C and lyophilized.
3b	TCA precipitate	Precipitate after TCA treatment of 3a was dialyzed against distilled H_2O and lyophilized.
4	Lipopolysaccharide	Cells + sonic vibration for 3 minutes + 10% CH_3COONa, 1% CH_3COOH, 1½ volume EtOH. Centrifuged 3000 rpm for 15 minutes. Precipitate dialyzed against distilled H_2O at 7° C and lyophilized.
5	Formamide	Cells + formamide, boiled for 1 minute. Acid alcohol added, centrifuged at 3000 rpm for 15 minutes. Precipitate discarded. Acetone added, precipitate dialyzed against distilled water at 7° C and lyophilized.
6a	Phenol, insoluble	Cells + two extractions with 88% phenol. Centrifuged at 3000 rpm for 15 minutes. Precipitate washed 3× with EtOH and dried *in vacuo*.
6b	Phenol, soluble	Supernatant fluids from 6a combined. White lipide layer at the surface was removed by separatory funnel and filtration. CH_3COONa + EtOH (2–3 vol.) added to precipitate polysaccharide. The precipitate was washed 3× with EtOH and dried *in vacuo*.
7a	Water and methanol, soluble	Cells + distilled H_2O. Sonic vibration for 3 minutes. Two volumes of CH_3OH-$CHCl_3$ (1:1) added. Brought to a boil and volume replenished with $CHCl_3$. Filtered through rapid filtering paper, distilled H_2O added to filtrate and mixed well. Water-alcohol layer removed in a separatory funnel, dialyzed against distilled H_2O at 7° C, and lyophilized.
7b	Chloroform, soluble	Chloroform layer from 7a dried *in vacuo*.

and ribonucleic acid, (RNA) the Dische diphenylamine reaction (*24*) and the Mejbaum orcinol method (*25*) were used, respectively.

RESULTS

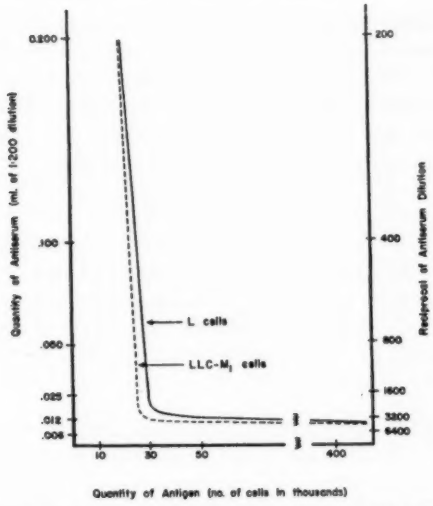
Cross-Reactivity Between Tissue Cells

Cross-reactions among 4 cell strains were studied by agglutination, complement-fixation, and cytotoxic reactions. The L and LLC-M_1 strains of mouse fibroblasts, HeLa, and Chang liver (human epithelial) cells were each found to produce high homologous antibody titers in rabbits (table 2). The 2 mouse fibroblasts cross-reacted completely with each other while exhibiting slight reaction with the human epithelial-cell lines, HeLa and Chang liver cells. Similarly, the HeLa and liver cell strains cross-reacted to a high degree while showing a much lower reaction with the fibroblasts.

TABLE 2.—Cross-reactions between tissue cells grown *in vitro* and their antiserums

Cell strain used as test antigen	Antiserum to cell strain	Reciprocal of complement-fixation titer	Reciprocal of agglutination titer
L	L	6, 400	640
L	LLC-M_1	6, 400	640
L	HeLa	800	20
L	Chang liver	400	20
LLC-M_1	L	6, 400	320
LLC-M_1	LLC-M_1	6, 400	320
LLC-M_1	HeLa	400	20
LLC-M_1	Chang liver	800	20
HeLa	L	400	20
HeLa	LLC-M_1	400	20
HeLa	HeLa	6, 400	320
HeLa	Chang liver	6, 400	640
Chang liver	L	800	20
Chang liver	LLC-M_1	800	<10
Chang liver	HeLa	6, 400	160
Chang liver	Chang liver	6, 400	320

To compare adequately two cells, such as the strain L and LLC-M_1 fibroblasts, which appear to demonstrate complete cross-reactivity, it is necessary to use varying concentrations of antigen and antibody. Rapport *et al.* (*26*) and de Almeida (*27*) have pointed out the usefulness of isofixation curves for a quantitative analysis of antigen-antibody systems by complement-fixation reactions. Increasing quantities of whole cells, L or LLC-M_1 fibroblasts, were added to increasing dilutions of rabbit antiserum (to the L cells). The titers obtained under such conditions were plotted as shown in text-figure 1. The close correlation between the curves for L and LLC-M_1 mouse fibroblasts at all points suggests no major antigenic differences between the two cell lines with respect to this antiserum.



TEXT-FIGURE 1.—Isofixation curves of strain L and strain LLC-M_1 cells with rabbit antiserum to strain L cells.

To demonstrate the cytotoxic activity of antiserum, the metabolic inhibition test was used. When strain L or strain LLC-M_1 mouse fibroblasts were treated with antiserum to the respective cells in the presence of guinea-pig serum, relatively high inhibition titers (1:5120) were obtained. These titers were higher than those detected by agglutination (1:640), but lower than the complement-fixation titers (1:7000). In the absence of guinea-pig serum, the inhibition titers were much lower (1:80). Cross-reactions as measured by the metabolic inhibition test revealed only slight reactivity (1:10 to 1:20) between either of the mouse fibroblast strains and antiserum to either of the human epithelial cell lines. The addition of guinea-pig serum alone or in combination with preimmune serum had no detectable cytotoxic effect on strain L or HeLa cells.

In absorption tests, both L and LLC-M_1 cell lines exhibited similar ability to remove complement-fixing antibodies from the antiserum to the L cell. Absorption of antiserums to the LLC-M_1 fibroblast gave similar results. On the other hand, adsorption of the L-cell antiserum with HeLa or Chang liver cells completely removed all complement-fixing antibodies to these cells, which resulted in an antiserum apparently specific for the mouse fibroblasts (L or LLC-M_1) and still of undiminished titer (1:3200).

Relationship of Nonspecific Antigens

In an investigation of serologic cross-reactions between tissue cells, one must consider the possible effect of nonspecific antigens absorbed from the medium or the presence of heterogenetic antigens associated with the cell. Horse serum was at first included as a necessary constituent of the

medium for shaken cultures. Later it was found that methylcellulose could replace horse serum when fibroblasts were grown in 199-peptone or YELP medium. For immunization purposes, one must consider that horse serum or other macromolecules in the medium may become adsorbed to the cell surface and be capable of stimulating the production of antibodies when whole cells are inoculated into animals. Tests were made for such antibodies and the results are presented in table 3. First it was demonstrated that L cells grown in the presence of horse serum and washed 3 times with Veronal-buffered saline still retained sufficient horse serum adsorbed to the cells to react with antibodies to horse serum. When the L cells were grown in the absence of horse serum (either in YELP or YELP + methylcellulose), no reaction was detected with antibodies to horse serum. Cells grown in serum-free medium had been carried in such medium for 1 year or more.

TABLE 3.—Test for the presence of antigens from medium components

Test antigen	Rabbit antiserum to	Reciprocal of titer by		
		Agglutination	Complement-fixation	Precipitation (ring test)
L cells (HS)*	Horse serum	40	3, 000	
L cells	Horse serum	<10†	<200†	
Horse serum‡	Horse serum		12, 800	10, 000
Horse serum‡	L cells (HS)*		3, 200	1, 000
Horse serum‡	L Cells		<200†	<10†
L cells	YELP + methylcellulose	<10†	<200†	
YELP + methylcellulose§	YELP + methylcellulose		<200†	<10†
L cells	L cells (HS)*	640	12, 800	
L cells	L cells	640	12, 800	
L cells (HS)*	L cells	640	12, 800	
L cells (HS)*	L cells (HS)*	640	12, 800	

* These cells were grown in medium containing horse serum.
† The lowest dilution of serum tested was negative.
‡ A 1:1000 dilution of horse serum was used for complement-fixation tests.
§ Medium was used undiluted for complement-fixation tests.

Horse serum was shown to react by complement-fixation and precipitation tests with antiserum to L cells grown in the presence of horse serum. No such reaction occurred with antiserum to cells grown in the absence of serum. No agglutination or complement-fixation reaction was observed between L cells and serum from 3 rabbits inoculated with the medium YELP + methylcellulose.

Investigation was then made to determine the effect of adsorbed horse serum upon the production of antibodies to the cell antigens. L strain cells grown in the absence of horse serum gave a comparable response (1:640 agglutination titer and 1:12,800 complement-fixation titer) with antiserum to cells grown either in the presence or absence of horse serum. Similarly, cells grown in the presence of horse serum gave an equivalent reaction with antiserum to cells grown either in the presence or absence of horse serum.

For the purpose of determining the possible heterogenetic nature of the surface antigens involved in the serologic reactions observed, tests were performed for the detection of blood group and Forssman's antigens. Agglutination titers of 1:20 were observed with both groups A and B human serums, which confirmed the findings of Goodman and Merchant (*28*), but no complement-fixation titers were obtained at serum dilutions of 1:200, lowest dilution tested, or greater.

The relationship of heterophile antigen and the Forssman antigen in mouse tissues has not been well established by other workers. In a hemolytic test, sheep red blood cells were incubated with L cell antiserum and guinea-pig serum (1:20) for 2 hours at 37° C. A hemolytic titer of 1:80 was found, but this was equivalent to the effect of normal serum. A complement-fixation test between Forssman antigen, an alcoholic extract of horse kidney (1:1000), and antiserum to L cells revealed no detectable titer.

Nature of Surface Antigens of Strain L Fibroblast

Effect of enzymes and chemicals upon surface antigens.—A number of enzymes that were readily available were chosen for screening to determine their effect on the agglutination of treated whole cells (strain L fibroblasts) with rabbit antiserum against untreated homologous cells. The effect of trypsin, in Veronal-buffered saline, *p*H 7.0, was difficult to evaluate. Frequently, treatment with a trypsin concentration of 0.25 percent or 0.50 percent, which has been used by other workers for obtaining cell suspensions, resulted in an agglomeration of cells in the saline control and occasionally in the higher dilutions of serum, even when incubation with enzyme was limited to about 5 minutes. A concentration of 0.10 percent trypsin usually resulted in satisfactory controls and an increase in agglutination titer of one to two tubes in a twofold dilution series. Similar results were obtained with pancreatin and with collagenase.

Lipase in a concentration of 1.0 percent was found to abolish an original titer of 1:320. Treatment with α-amylase resulted in a slight increase in titer, from 1:320 to 1:640. The effects of other enzymes tested are presented in table 4. Several of these are extremely crude and contain mixtures of enzymes.

Other enzymes tested without a significant change of agglutination titer included β-amylase and hyaluronidase. Treatment with trypsin (2 × crystalline), papain, and chymotrypsin resulted in an agglomeration of cells.

Treatment of the L cells with 0.001 M sodium periodate resulted in a complete loss of an agglutination titer of 1:640.

Fractionation of whole cells.—Several widely used chemical procedures were initially used to fractionate the tissue cells and so obtain extracts with varying proportions of protein, carbohydrate, and lipide. When rabbit antiserum to these fractions was reacted with the whole cell, agglutination and complement-fixation titers were found as recorded in table 5. Although most of the extracts were prepared early in the work when cells

TABLE 4.—Agglutination titers of strain L mouse-cell antiserum with strain L cells after enzymatic or chemical treatment of the cells

Agents used for treatment of cells	Reciprocal of titer before treatment	Reciprocal of titer after treatment
0.1% Trypsin (Difco, 1:250)	640	1, 280
1.0% Lipase*	320	<10
0.1% α-Amylase†	320	640
0.1% Pancreatin‡	160	640
0.1% Pancreatin§	160	320
0.1% Lysozyme‖	320	160
0.1% Collagenase¶	160	320
0.001 M Na periodate	640	<10
0.0001 M Na periodate	160	160

*Wheat-germ lipase, Worthington Biochemical Corp., Freehold, N.J.
†Nutritional Biochemical Corp., Cleveland, Ohio.
‡Difco Laboratories, Inc., Detroit, Mich.
§Parke, Davis & Co., Detroit, Mich.
‖Armour & Co., Chicago, Ill.
¶Dr. John J. MacLennan, Columbia University, New York, N.Y.

were grown in the presence of horse serum, the agglutination and complement-fixation tests were performed with cells grown in the absence of serum. Several water-methanol extracts were prepared from L cells grown in the absence of serum.

Relatively high complement-fixation titers were produced with many of the extracts. No reactions were observed with normal serum taken from the rabbits prior to inoculation. Each antiserum given in table 5 represents a single rabbit that was inoculated.

TABLE 5.—Agglutination and complement-fixation titers of strain L mouse cells with antiserums to cell extracts

Extract No.	Nature of extract	Reciprocal of agglutination titer	Reciprocal of complement-fixation titer
1	Aqueous	20	1, 600
2a	0 to 20% $(NH_4)_2SO_4$ precipitate	40	800
2b	20 to 30% $(NH_4)_2SO_4$ precipitate	20	3, 200
2c	30 to 40% $(NH_4)_2SO_4$ precipitate	40	6, 400
2d	40 to 50% $(NH_4)_2SO_4$ precipitate	40	1, 600
2e	Supernatant from 40 to 50% $(NH_4)_2SO_4$ precipitate	<10	800
3b*	TCA precipitate	40	6, 400
4	Lipopolysaccharide	20	1, 600
5	Formamide	40	800
6a	Phenol, insoluble	20	3, 200
6b	Phenol, soluble	40	800
7a	Water-methanol, soluble	640	1, 600

*Insufficient amounts of extract 3a were obtained to perform immunization experiments.

The water-alcohol fraction of the methanol-chloroform extract #7a was selected for more intensive investigation. While several other fractions exhibited equal or higher complement-fixing titers, the unusually high agglutinating titer of this fraction suggested a closer relationship to

the cell surface. In preparation, practically all the protein was denatured and removed by filtration. The chloroform soluble lipide fraction also was removed. The soluble fraction remaining was free from whole cells and any visible fragments. Presumably it contained glycolipides among other components. The elimination of protein antigens that would be difficult to separate and study was thought to be an advantage at this stage of investigation. The method of preparation used resulted in a yield of approximately 1 percent of the dry weight of the cell.

Absorption of antibody against the water-methanol extract with strain L mouse fibroblasts removed all complement-fixing antibody to the homologous cell. However, absorption with HeLa and Chang liver cells (human epithelial) removed only complement-fixing antibody to these cells and did not alter the titer to either of the mouse fibroblasts. There was no evidence of an increase in cell specificity of the antiserum prepared to the water-methanol extract over that prepared against whole cells, since it reacted equally with either L or LLC-M_1 fibroblasts.

The cytotoxic effect of water-methanol extract antiserum for strain L cells was tested by metabolic inhibition in the presence of guinea-pig serum and was found to occur at a dilution of 1:160; the complement-fixation titer of the serum was 1:1600. The cytotoxic effect of the antiserum could be removed by absorption with whole L cells.

The ability of antiserum to the water-methanol extract to react in agglutination and complement-fixation reactions and to show cytotoxic effect against the whole cell, plus the ability of these reactive components to be adsorbed by the homologous cell line, indicates strongly that the extract contains a surface antigen(s) of the strain L fibroblast.

Attempts to demonstrate reactivity between cell extracts and antiserums to whole cells or antiserums to extracts have been unsuccessful. The tests attempted included precipitation, complement-fixation, hemagglutination, blocking of agglutination, and absorption.

The results of several chemical determinations made upon the lyophilized whole cells and extracts taken from these cells are presented in table 6. The major increase of material in the water-methanol extract appeared to be due to hexosamine. The ultraviolet absorption spectrum of this extract showed a peak at 260 mμ. Since nucleic acids are not soluble in

TABLE 6.—Chemical tests performed on the strain L mouse fibroblasts and on a water-methanol extract

Test	Analysis		Relative percentage increase (percent)
	Whole cells (percent)	Extract (percent)	
Nitrogen	9.0	9.0	0
Hexose	1.7	6.6	288
Hexosamine	0.6	4.2	600
DNA	5.5	21.2	285
RNA	9.2	39.1	325

methanol-chloroform, the compounds giving a positive reaction for RNA and DNA and showing absorption at 260 mμ may be nucleosides or other low molecular weight compounds.

DISCUSSION

Many current problems in research using cell culture involve a need for classifying and distinguishing cell strains. The problems of accidental cell contamination or mixture of cell lines have been emphasized by several workers (*3*, *7*). Attempts to distinguish tissue-cell strains immunologically with the use of whole cells (*2*, *3*) or nucleoprotein fractions (*4*) have not been successful. To date no generalized test has been developed for characterization of cell strains. Brand and Syverton (*7*) have used a hemagglutination test that offers promise in distinguishing tissue cells from different species. However, with the many attempts to differentiate tissue cells, no basic effort has been made to analyze a given cell line both chemically and immunologically.

The present work has sought to clarify this problem by beginning an analysis of chemically prepared fractions of an established cell line. The approach used in this study was to emphasize the nature of the cell surface. In many microorganisms, fractionation of cells has yielded antigens with a wide range of group or species reactivity. In pneumococci, as well as other microorganisms, surface antigens have been found to be type-specific while less superficial antigens have been group-specific or broadly cross-reactive. With tissue cells grown *in vitro*, it appears that the initial reaction with homologous antiserums does occur at the cell surface (*5*). Also *in vivo*, it is likely that such superficial antigens are primarily involved in antigen-antibody reactions before the more deep-seated antigens react. It would appear then that the surface antigens would be a logical choice for initiating an investigation of specific differences.

Due to limitations in technology, the agglutination test was chosen to narrow the reactions to surface antigens of whole cells. Complement-fixation and cytotoxicity-metabolic inhibition tests have been used to support these observations and to give additional information. In the results reported, cross-reactions were demonstrated by these serologic tests between the 2 fibroblasts from mouse tissue or between the 2 epithelial cells from human tissue. Because of the source of the cells, these reactions might be attributed to species antigens.

Differences in growth rate, metabolism, and nutritional response characterize the L and LLC-M_1 strains as distinct cell lines (*29*). These differences are quite distinct and have been consistent throughout the long period of cultivation in this laboratory. In spite of this fact no serologic differences could be demonstrated.

For evaluating the cytotoxic effect of antiserum, the metabolic inhibition test proved to be a very useful technique for observing, grossly, the cessation of active metabolism, and, microscopically, the morphological

damage to cells. Antiserums to tissue cells grown *in vitro* have been produced by many workers. In general the cytotoxic titer of such serums has been relatively low, 1:100 or less. In the experiments reported here, cytotoxic titers of strain L antiserums were found to be high (1:5120) in the presence of guinea-pig serum. These antiserums also exerted a cytotoxic effect (1:80) without the addition of guinea-pig serum. No serum was used for the growth of these cells, but it is not known whether this cytotoxicity may be an effect of the antibody itself or antibody plus a heat-stable component of rabbit serum. The latter possibility is suggested by the findings of Björklund (*30*).

The elimination of serum from the growth medium of the strain L and LLC-M_1 mouse fibroblasts was an important step in these studies, since such nonspecific protein can be adsorbed by tissue cells, which result in a possible blocking of specific cell antigens, or, on inoculation of these cells into animals, can initiate the production of serum antibodies. The removal of serum was also of value because of the possibility of contamination with noncytopathogenic viruses or bacteria, pleuropneumonia-like organisms, or toxic substances.

The treatment of tissue cells with enzymes and chemical agents utilized an approach that has been successful in the study of the surface of the red blood cell (*31*, *32*). The agglutination of strain L cells in the presence of homologous antiserum was completely removed by treatment of the cells with periodate. The presence of polysaccharide in whole cells and in the water-methanol extract was indicated by a positive test for hexose and hexosamine. The periodate ion is known to inactivate the blood group (*33*) and other polysaccharide-containing antigens, and this action may be responsible for the elimination of the agglutination titer. However, periodate also oxidizes the hydroxyamino acids and has some inactivating effect on several biologically active proteins (*34–36*). Such action is usually slower than the effect on polysaccharide, but caution must be used in interpreting the effect of periodate on an unknown chemical structure.

The failure of trypsin to decrease the agglutination titer appeared to minimize the importance of some peptide units as major antigens of the cell surface. The importance of lipide at the cell surface was demonstrated in the action of lipase, which completely eliminated the agglutination titer with homologous antiserum. A slight increase in agglutination titer after the use of α-amylase, if significant, may have been due to the removal of some polysaccharide groupings, which resulted in the exposure of subsurface antigens.

After the fractionation of the strain L mouse fibroblast, antibodies were obtained by immunization with each of the fractions prepared. Most of these antiserums were capable of agglutinating whole cells. Some lipopolysaccharide is closely linked with protein and is precipitated with it upon addition of trichloroacetic acid or ammonium sulfate. For this reason emphasis was placed upon the characterization of the water-methanol extract. Here the elimination of much of the protein and

chloroform-soluble lipide offered the initial advantage of studying a more select group of antigens. From the method of preparing the water-methanol extract, it appeared that polysaccharide alone or in some combination with lipide was the major active serologic grouping on the cell surface. This seems to corroborate the observations with regard to loss of agglutinating capacity after treatment with lipase or periodate. Nucleosides or other low molecular weight compounds are also present in the extract and could contribute to the chemical structure of the surface antigens.

Antiserum to the water-methanol extract showed no evidence of increased specificity, for it reacted equally well with both the L and LLC-M_1 mouse fibroblasts. It may be that protein is essential for cell specificity. Also it is possible that the emergence of common antigens in these long-term cultured cells, as suggested by Coriell (*3*), could be responsible for failure to detect antigenic differences.

The results obtained from a combination of enzymatic studies and the analysis of chemical extracts suggested the importance of carbohydrate groupings on the cell surface. Protein groups on the surface or in a subsurface location seemed to be of lesser importance in the serologic reactions observed, though they may be of great importance in cell specificity. Lipide was important to the antigenic structure of the cell membrane, but it is not known whether its importance may reside in its position as a structural component or in the role of lipide haptenes or lipopolysaccharides.

It is difficult to explain the failure to demonstrate a serologic reaction between the water-methanol extract and antiserum to either the whole cell or the extract. It is possible that the concentration of antigen in the extract is extremely small. However, it may be that antigenically reactive components are bound in some way in the extract or are altered during lyophilization so that they are not free to react with homologous antiserum. Upon inoculation into rabbits and after enzymatic or chemical splitting of linkage or covering groups, these sites would again be free to initiate antibody production.

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Effect of Phenazine di-*N*-oxide and Phenazine on Total Cellular Dry Mass of Mouse Ehrlich Ascites Cells as Measured by Interference Microscopy [1, 2]

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SUMMARY

Eighteen Swiss-Webster mice were inoculated with equal quantities of Ehrlich ascitic fluid and divided into 3 groups of 6 animals each. The first group was untreated as control. The second group was treated with phenazine, a chemical which has little effect on Ehrlich ascites tumor, as a negative control. The third group was treated with phenazine di-*N*-oxide, a newly reported carcinostatic agent derived from phenazine. Cell samples were taken daily for cellular dry-mass measurements with interference microscopy. All the phenazine di-*N*-oxide-treated animals responded to the treatment with a quick and progressive increase in cellular dry mass and size and with the number of a certain type of small cells. Two of the animals in the phenazine-treated group showed similar, but much milder, reactions to the treatment. The control group showed no such reaction.—J. Nat. Cancer Inst. 26: 435–443, 1961.

PHYSICAL OR chemical agents detrimental to cells may alter their total dry mass. By use of immersion refractometry, Barer and Joseph (*1*) showed that the cytoplasmic concentration of the mouse lymphocytes changed after X irradiation. The modal concentration dropped rapidly immediately after the irradiation and gradual recovery began within the next 2 to 3 days. Elson (*2*), using Barer's technique, demonstrated the same phenomenon in mouse lymphocytes after treating the mice with CB1348, a nitrogen mustard derivative. Easty *et al.* (*3*) treated Ehrlich and "active" Landschutz mouse ascites cells with ribonuclease and found an initial increase in the cellular mass during the treatment and a loss of mass immediately thereafter. Caspersson *et al.* (*4*) treated three different lines of Ehrlich ascites tumor with a whole-body dose of 1250 r and also found considerable dry-mass increase in the cells. The interference microscope was used for the last two. We (*5*) too have demonstrated by interference microscopy that mouse Ehrlich ascites cells treated with rabbit antiserum undergo a loss of about 50 percent of their dry cell mass with a marked increase in the cell area.

[1] Received for publication August 15, 1960.

[2] This work was supported by a research grant from the Damon Runyon Fund and grant T-134 from the American Cancer Society, Inc.

Dry cell-mass studies might be profitably used in the search for chemotherapeutic agents used against cancer cells. The present report concerns the use of the interference microscope as a valuable instrument for the quantitation of dry cell mass after treatment of Ehrlich ascites cells with an active carcinostatic agent. Phenazine di-*N*-oxide, a phenazine derivative, and phenazine were selected for our first study because Furst *et al.* (*6*) found the former the most effective for mouse Ehrlich ascites tumors. More than 90 percent of tumor-bearing animals treated with this compound by intraperitoneal injections survived 30 days or more and were then free of tumors, while there was an average 13-day survival of the untreated control group. Animals treated with phenazine were unaffected by it, and had an average survival time of 15 days. Thus phenazine was chosen to serve as a negative control in our study. It was found later, however, that this chemical had a mild effect on the dry mass of the untreated cells.

MATERIALS AND METHODS

Treatment schedule.—Swiss-Diablo mice were randomized and divided into 3 groups of 6 mice each. All animals were inoculated intraperitoneally with 0.1 ml ($10–16 \times 10^6$ cells) of undiluted ascitic fluid from a donor mouse bearing a 7-day-old Ehrlich ascites carcinoma (diploid). Group 1, the control animals, was untreated and group 2, the negative control animals, was given a suspension of phenazine in normal saline intraperitoneally. The first 3 mice were treated with 1.5 mg/mouse/day $\times$ 5; the second 3 mice were given twice that amount. Group 3 was handled similarly to group 2, but phenazine di-*N*-oxide was used in place of phenazine. Treatment was started 5 days after the inoculation of the tumor and continued for 5 days or until the animal died. Changes in cells were followed during the 1st day of treatment by withdrawing fluid and examining cells every hour for 6 hours and during the rest of the lifespan of the animal by withdrawing fluids and examining cells once daily.

Delayed therapy on tumor growth in mice.—By the procedure previously reported (*6*), 7 groups of 10 animals each were given tumor. Controls were treated with carboxymethyl cellulose (CMC). At intervals of 0, 1, 2, 3, 5, and 7 days following the tumor injection, therapy began. The dose was 1.5 mg/mouse/day for 5 consecutive days.

Method of examining cells.—The cell-sample slide for interference microscopy was prepared in the following manner for all three groups: One small drop of ascitic fluid was aspirated from the animal with a 25-gauge needle on a 2 ml syringe. A small quantity of the aspirated fluid was evenly suspended on a slide in 2 drops of saline. A coverglass was immediately put over the suspension. A gentle pressure was exerted on the coverglass with a lens paper folded into a 16-layer thick pad to absorb the excessive liquid and to flatten the cells slightly without injuring them. The slide was then put under the interference microscope for dry-mass

determination. The first 30 cells that were not clumped or deformed were measured for the individual total dry-mass value of each cell. In the controls, group 1, the first sample of the cell population of each mouse was taken when the tumors were 5 days old. Subsequently one sample was taken daily throughout the lifespan of each animal.

For groups 2 and 3, after the tumor was 5 days old, a sample of fluid was drawn and examined. This permitted a control on each animal before administration of the drug. Drugs were then started the same day. On the following 4 days, cell samples were drawn before the drugs were administered. In other experiments the drug was administered to mice with 8-day-old tumors. To ascertain early cell changes, if any, fluid was aspirated every hour from mice for 6 hours on the 1st day of treatment. These cells also were measured by the use of the interference microscope.

Calculations of dry cell mass per cell.—The A-O Baker interference microscope in our experience has a sensitivity of 3° on the halfshade-eyepiece scale which is equivalent to $\frac{1}{120}$ wavelength.

Our previous work (*5*) with Ehrlich ascites cells revealed that by measuring the same 50 cells at 4 different intervals, 0, 30, 90, and 180 minutes with the Baker interference microscope, normal cells suspended in saline with a small quantity of rabbit and guinea-pig serums showed an average dry-mass loss of 2.2 percent at 30 minutes, and 4.6 percent at 90 and at 180 minutes, from their original values. The agreement between the losses at 90 and 180 minutes gives a good indication of the accuracy of the interference microscope we used.

The dry mass was computed according to the following formula:

$$M = A\emptyset/x$$

Where A = area in square centimeter, $\emptyset$ = optical path difference between the cell and its surrounding medium, and x = specific refractive increment (0.18 for most biological materials).

RESULTS

The cellular dry-mass data were tabulated and experimental results are presented. No noticeable changes took place in cell size or mass weight during the 1st day of treatment. The values for these cells paralleled those of the untreated controls. In the control group, table 1, a Gaussian distribution of cell-mass dry weight was noted. The range of values spread from 28.8 to 1300 arbitrary units, with the mode being between 200 and 400 units. The frequency distribution of dry mass varied from day to day, but only within narrow limits. Table 2, the negative control group, shows that phenazine either had no, or only minimal, effect on cellular dry mass. Only animal #3, treated with 1.5 mg/mouse, and animal #4, given 3 mg/mouse, had tumor cells that indicated a slight

TABLE 1.—Control group (each daily sample = 30 cells)

DAYS AFTER TUMOR TRANSPLANT	NUMBER OF CELLS	CELL DRY MASS IN 100 ARBITRARY UNITS * (5 / 10 / 15 / 20 / 25 / 30 / 31-76)	MEAN	σ + −
5		4 13 7 3 2 1	316.7	137.89
6		6 5 10 6 3	235.0	113.58
7		3 1 13 4 5 4	308.3	139.25
8		3 5 8 9 4 1	285.0	116.47
		ANIMAL N° 1 DOSAGE: NONE		
5		2 5 15 3 4 1	266.7	108.85
6		2 6 6 10 2 2 1 1	313.3	173.06
7		2 2 15 9 1 1	281.7	118.13
8		1 4 11 9 4 1	306.7	132.59
		ANIMAL N° 2 DOSAGE: NONE		
5		3 8 17 1 1	221.6	85.57
6		1 9 11 6 1 2	265.0	107.54
7		4 1 14 9 1 1	283.3	127.86
8		1 3 17 8 1	276.7	87.07
9		2 1 19 5 2 1	271.7	98.26
10		2 2 6 13 6 1	326 7	109.90
		ANIMAL N° 3 DOSAGE: NONE		
5		2 3 10 11 4	295.0	99.67
6		1 5 12 10 1 1	276.7	92.63
7		4 15 10 1	263.3	97.20
8		2 2 15 8 2 1	275.0	93.99
9		1 5 17 6 1	255.0	73.71
10		3 1 15 6 2 2 1	311.7	204.09
11		4 3 7 12 1 2 1	291.7	129.31
12		1 4 10 10 4 1	308.3	114.99
13		4 4 9 12 1	253.3	113.05
		ANIMAL N° 4 DOSAGE: NONE		
5		3 1 17 8 1	258.3	92.50
6		1 14 12 2 1	315.0	97.81
7		5 16 6 2 1	286.7	113.05
8		3 13 9 3 1 1	308.3	103.54
9		2 2 13 9 1 1 1 1	315.0	151.87
10		3 5 12 10	258.3	95.16
11		2 4 13 7 2 1 1	288.3	141.97
12		1 9 10 9 1	250.0	90.14
13		2 6 13 8 1	245.0	85.25
14		2 11 14 3	216.7	71.98
15		2 7 11 8 1 1	261.7	113.97
		ANIMAL N° 5 DOSAGE: NONE		
5		1 15 7 1 2 3 1	351.7	177.83
6		1 7 10 10 1 1	281.7	107.81
7		4 9 15 2	301.7	76.08
8		5 14 8 2 1	303.3	142.41
9		1 8 5 13 1 1 1	288.3	118.98
10		2 3 12 7 3 1 2	303.3	137.65
11		3 2 9 11 1 1 1 1 1	335.0	201.82
12		6 7 14 3	296.7	86.30
13		1 1 16 12	273.3	59.84
		ANIMAL N° 6 DOSAGE: NONE		

* One arbitrary unit equal to 1×10^{-12} g.

reaction to the drug. This was manifested by a slightly wider spread than the controls, because of an increase of number of larger cells.

Table 3, the phenazine di-*N*-oxide-treated group, shows that all animals, regardless of dose, showed progressive reaction to the phenazine di-*N*-oxide. The reaction generally started on the 2d or 3d day after the drug was administered; this corresponded to 7 to 8 days after the inoculation of the tumor cells. The frequency distribution of dry cell mass changed. A marked increase of cells was noted with mass much beyond that of the limits of the untreated controls. Many cells continued to increase in size and mass, even after the withdrawal of treatments.

An apparent increase of small cells with cellular dry mass of between 0 and 150 arbitrary units was also seen. This was most pronounced in

TABLE 2.—Group 2: phenazine treated (each daily sample = 30 cells)

DAYS AFTER TUMOR TRANSPLANT	NUMBER OF CELLS	CELL DRY MASS IN 100 ARBITRARY UNITS *	MEAN	σ +−
		5 10 15 20 25 30 31-76		
5	1	1 10 15 2 1 1	341.7	121.34
6	4	9 14 2 1	325.0	177.01
7		3 14 9 3 1	295.0	101.32
8	2	1 1 11 4 1	316.7	119.78
9	2	7 7 6 3 1 2 1 1	420.0	208.31
10	2	2 10 7 6 3	325.0	121.11
11	4	3 4 7 6 3 1	345.0	172.53
12	4	2 9 6 3 2 2 1 1	343.3	185.51
		ANIMAL N° 1 DOSAGE: 1.5 mg.		
5		1 10 16 2 1	325.0	101.65
6	2	3 10 11 1 2 1	315.0	169.51
7	1	3 6 10 5 2 1	335.0	120.69
8	1	4 13 8 2 1 1	291.7	108.27
9	4	2 11 11 1 1	275.0	108.01
10	3	1 9 9 4 1 1 1	356.7	212.72
11	1	2 13 11 2 1	306.7	107.61
		ANIMAL N° 2 DOSAGE: 1.5 mg.		
5	4	5 12 6 2 1	268.3	151.51
6	4	5 11 4 1 2 1 1 1	320.0	246.42
7	1	8 9 8 2 1 1	311.7	218.68
8	5	4 13 6 1 1	275.0	243.58
9	6	4 13 5 1 1	248.3	164.18
10	4	10 12 3 1	218.3	114.55
11	3	5 13 6 1 1 1	280.0	168.00
12	4	5 5 14 2	275.0	111.80
13	3	9 13 4 1	225.0	84.66
14	4	11 6 8 1	241.7	132.50
		ANIMAL N° 3 DOSAGE: 1.5 mg.		
5	1	2 15 9 2 1	285.0	91.65
6	4	15 8 3 1 1	275.0	132.28
7	3	2 11 9 3 2	301.7	114.55
8	5	3 10 7 4 1	353.3	157.94
9	1	1 4 9 9 1 2 2 1	555.0	636.82
10	8	4 9 6 1 1 1	323.3	251.82
11	1	3 10 12 3 1	323.3	189.95
12	3	2 5 13 1 1 2 2 1	473.3	610.39
13	3	1 12 9 1 1 1 1 1	373.3	311.04
		ANIMAL N° 4 DOSAGE: 3.0 mg.		
5	2	8 11 7 1 1	258.3	115.71
6	1	2 15 10 2	281.7	81.38
7	4	5 11 8 1 1	261.7	134.74
8	5	7 11 8 1	221.7	111.75
9	3	3 18 4 1 1	268.3	143.02
10	4	7 11 5 1 2	248.3	124.99
11	5	7 12 5 1	220.0	93.41
12	2	13 7 5 2 1	233.3	119.78
13	2	13 11 3 1	235.0	133.17
14	1	7 11 5 3 3	268.3	132.87
15	1	9 10 8 2	261.7	93.04
16	2	15 8 2 2 1	221.7	130.98
		ANIMAL N° 5 DOSAGE: 3.0 mg.		
5		5 14 11	273.3	61.21
6	1	2 12 12 2 1	291.7	98.60
7	4	1 14 7 2 2	276.7	139.93
8	2	1 14 10 1 2	286.7	106.99
9	2	12 9 5 2	315.0	112.10
10	3	4 10 10 3	276.7	104.47
11	1	4 18 3 4	270.0	85.97
12	1	7 12 4 4 2	298.3	156.38
13	4	8 11 3 3 1	240.0	114.86
		ANIMAL N° 6 DOSAGE: 3.0 mg.		

* One arbitrary unit equal to 1×10^{-13} g.

the treated animals. The increase of dry weight may reflect inhibition of cell division.

For a comparison the data were arranged into percentage histograms (text-fig. 1). The results from all the daily samples in each group were

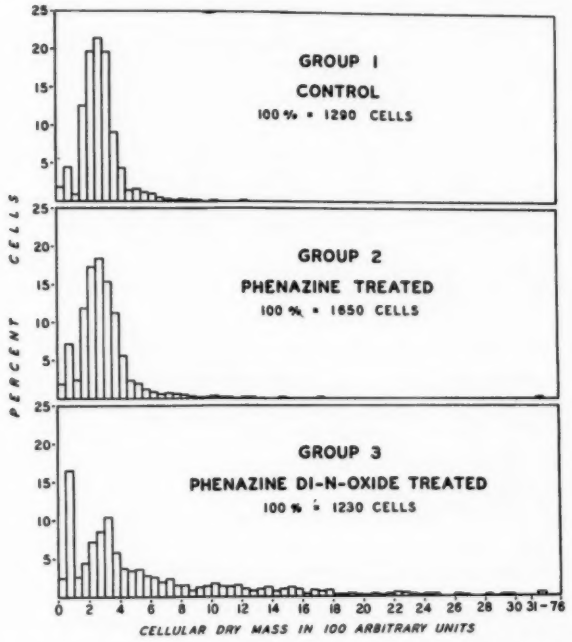
TABLE 3.—Group 3: phenazine di-*N*-oxide (each daily sample = 30 cells)

DAYS AFTER TUMOR TRANSPLANT

CELL NUMBER	CELL DRY MASS IN 100 ARBITRARY UNITS *	MEAN	σ +−
5	11 15 3 1 — ANIMAL N° 1	331.7	70.40
6	2 3 1 7 12 2 2 1 — DOSAGE: 1.5 mg.	488.3	153.80
7	10 8 1 2 1 1 2 3 1 1	306.7	287.08
8	8 3 2 1 2 1 2 2 3 2 2 1 1	616.7	491.44
9	7 5 1 2 2 1 1 2 1 1 3 2 1 1	741.7	741.99
10	7 4 4 1 1 1 2 1 1 1 1 2 1 1 1	655.0	685.15
5	2 10 15 3 — ANIMAL N° 2	323.3	94.41
6	1 9 16 3 1 — DOSAGE: 1.5 mg.	326.7	71.28
7	3 5 11 5 4 1 1	386.7	193.08
8	3 6 9 6 2 2 1 1	405.0	232.59
5	2 11 13 3 1 — ANIMAL N° 3	315.0	80.00
6	2 2 5 12 2 2 2 2 1	455.0	482.32
7	5 6 9 5 3 1 1 — DOSAGE: 1.5 mg.	358.3	211.47
8	6 3 5 5 4 1 5 1	338.3	211.32
9	9 1 1 4 1 6 3 3 1 1	480.0	326.17
10	7 1 1 2 3 4 3 2 3 1 1 1 1	590.0	387.76
5	1 1 22 5 1 — ANIMAL N° 4	256.7	59.84
6	3 1 6 8 4 2 2 1 1 1 1 — DOSAGE: 3.0 mg.	508.3	289.64
7	6 1 4 6 6 1 3 1 1 1	628.3	336.88
8	9 2 1 2 2 1 1 4 6 1 1	661.7	486.30
9	7 2 1 1 1 1 3 2 2 1 2 4 1 1 1	1096.7	1347.82
10	8 1 1 2 3 3 5 2 2 1 2	940.3	604.75
11	2 1 1 2 1 3 1 5 5 3 2 2 1 1	1105.0	441.89
5	7 17 5 1 — ANIMAL N° 5	253.3	91.00
6	1 7 14 7 1 — DOSAGE: 3.0 mg.	255.0	96.26
7	4 1 7 11 6 1	308.3	134.99
8	6 2 6 10 1 3 1 1	301.7	171.14
5	4 9 11 1 4 1 — ANIMAL N° 6	338.3	153.26
6	7 6 11 4 1 1 — DOSAGE: 3.0 mg.	241.7	239.91
7	5 1 4 12 3 2 1 1 1	350.0	192.68
8	10 2 2 3 6 3 1 2 1	330.0	243.70
9	3 1 2 5 12 3 1 3	523.3	210.35
10	14 1 1 2 3 4 2 2 1	391.7	348.89
11	8 1 1 2 2 1 1 8 3 3	703.3	458.20
12	9 4 1 1 1 5 3 1 3 2 1	618.3	514.58
13	4 1 1 1 1 3 2 2 2 2 4 4 1 1 1	1271.7	726.05
14	9 4 2 1 1 1 2 2 1 2 1 2 1 1	731.7	691.83
15	1 1 3 1 1 3 2 3 1 4 2 1 1 2 1 1 1	1543.3	814.91
16	14 5 1 2 1 1 2 2 1 1	786.7	931.99
17	11 1 3 2 1 2 1 1 1 1 1 1 3 1	960.0	1048.38
18	21 2 2 1 2 1 1	503.3	887.85

* One arbitrary unit equal to 1×10^{-13} g.

pooled. The percentages of cells of similar dry-mass values were plotted against their cellular dry mass in arbitrary units. From this text-figure it can be seen that: (*a*) There is a peak representing the small cells within the 150 arbitrary-units range in all 3 histograms. This suggests that most of these cells are of a type different from the tumor cells. They are possibly lymphocytes which are undistinguishable from the ascites cells of the same size range under the interference microscope. These cells are present in a very small number in the control group, slightly increased in number in the negative control group, and greatly increased in number in the experimental group. This may reflect the scarcity of tumor cells in animals treated with the active agent. (*b*) Of 1,230 cells measured in the experimental group, 30 percent had more than 600 arbitrary units of dry mass, while only 3.9 in the negative control group and 2.5 percent in the control group had more than this number.


GROUP 1
CONTROL
100 % • 1290 CELLS
GROUP 2
PHENAZINE TREATED
100 % • 1650 CELLS
GROUP 3
PHENAZINE DI-N-OXIDE TREATED
100 % • 1230 CELLS
PERCENT CELLS
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 31-76
CELLULAR DRY MASS IN 100 ARBITRARY UNITS

TEXT-FIGURE 1.—The percentage histograms represent the pooled results from all the daily samples in each group. The percentages of cells of similar dry-mass value were plotted against their dry mass in arbitrary units. The peaks on *left* of each histogram suggest that they represent some small non-tumor cells. Thirty percent of the cells in the experimental group had more than 600 arbitrary units of dry mass, while only 3.9 in the negative control group and 2.5 percent in the control group had more than this number.

These summarized histograms, however useful they are, cannot substitute the tabulation charts of the individual samples, since the reaction of each animal to phenazine di-*N*-oxide can be demonstrated only by individual charts of daily samples. Neither can the continuous effect of phenazine di-*N*-oxide after termination of treatment be illustrated by the summarized histograms as in the individual charts of the daily samples.

This experiment was not designed to study the survival time of the treated animals, but rather the reaction caused by the chemical agents at cellular levels. However, since the drugs were administered after the tumors were well developed, in contrast to the chemotherapeutic studies (*6*), data are presented on survival time with delayed therapy (table 4). In spite of the change caused in cells by the drug, there is no increase in survival time when tumorous animals are treated after maximal growth of tumor cells.

The mean dry mass and standard deviation (SD) for each daily sample were calculated. They vary a great deal from day to day even in the control group, mostly because of the occasional appearance of the very

TABLE 4.—Effect of delayed therapy on survival time

Group*	Days after tumor inoculation	Percent survival after day: 7	14	21	30
I	Control	90	20	0	0
II	0	100	100	100	100
III	+1	100	90	90	90
IV	+2	100	100	80	80
V	+3	100	100	90	70
VI	+5	100	80	20	10
VII	+7	90	60	10	0

*There were 10 animals in each group. Group I, control, was treated with CMC. Groups II to VII were treated with phenazine di-*N*-oxide with a dose of 1.5 mg/mouse/day for 5 consecutive days after inoculation, beginning at 0, 1, 2, 3, 5, and 7 days, respectively.

large and very small cells. One large cell may have much effect on the value of the mean and SD. The individual means and SD's may have little meaning in the group in this case, but when the mean of the means of each group and the ranges of means and SD's of the three groups are compared, they further illustrate the differences among the three groups. Thus, the mean of the means of group 1, the control, is 284.3. That of group 2, the negative control, is 297.7, while that of group 3, the phenazine di-*N*-oxide treated, is 554.3; the means of the control group range from 216.7 to 351.7 units and SD's range from ± 59.84 to ± 204.9; the means of the negative group range from 220.0 to 555.0 units and SD's range from ± 61.21 to ± 836.82, and the means of the experimental group range from 241.7 to 1543.3 units and SD's range from ± 59.84 to ± 1347.82.

DISCUSSION

In an attempt to apply cellular dry-mass studies to cancer chemotherapeutic investigations, we found that the result is rather encouraging—at least with these drugs. Our results are in complete agreement with the information obtained with survival-time tests by Furst *et al.* (*6*). They found that over 90 percent of the phenazine di-*N*-oxide-treated animals survived 30 days or more, while the average survival time of the phenazine-treated animals was 15.3 ± 1.8 days and that of the untreated controls, 13.3 ± 2.0 days. Our results show that all the animals treated with phenazine di-*N*-oxide responded quickly and progressively by increase in cellular dry mass, while only a mild response was found in 2 animals treated with phenazine. The mean of means and the range of means of each group are in good correlation with the mean survival time of each group (*see* Results). Phenazine, though ineffective as a carcinostatic agent, is not entirely inoculous to the Ehrlich ascites cells.

The response of the phenazine di-*N*-oxide-treated animals to the two different doses, 1.5 mg/mouse and 3 mg/mouse, showed no appreciable difference in our results.

The average survival time of our animals in the cell studies was not prolonged among the two drug-treated groups. Our treatment was started on the 5th day, *i.e.*, during the peak of tumor growth, while in the thera-

peutic studies the treatment was started between 24 to 48 hours after tumor inoculation. We started the drug administration late for cell studies because the Ehrlich cell population in the peritoneum was too small for easy sample-taking during the first 48 hours. Evidently, when treated late, phenazine di-*N*-oxide cannot stop the tumor growth, but the effect of the drug can still be clearly demonstrated at a cellular level.

The accuracy of this study can only be within the limits of reproducibility of interference microscopy. Davies *et al.* (*7*) found that interference microscopy gave results in agreement to about ±15 percent with the results obtained from soft X-ray absorption method on a variety of biological objects. Barer (*8*) measured the human red cells by interference microscopy and reported the average dry-mass value per cell as 31.4×10^{-12}g, which was in good agreement with the hemoglobin values obtained by the well-known clinical method, $27–32 \times 10^{-12}$g. Homogeneity of the biological material under investigation is essential in interference microscopy. When they are slightly flattened, the living ascites cells are satisfactorily homogenous, except that a few small, refractive spheres were usually on the surface of the cells in both treated and untreated groups. These spheres were not taken into account in our measurements. Since they are generally in both treated and untreated cells on the average in equal numbers, the results of the experimental and control groups would not be affected.

For convenience, we used arbitrary instead of absolute dry-mass unit as a quantitative indicator, one arbitrary unit equal to 1×10^{-12} g. As a whole, the results of our work are consistent, and the increase in cellular dry mass in our phenazine di-*N*-oxide-treated group is unmistakable.

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Bovine Ocular Squamous-Cell Carcinoma. IV. Tissue-Culture Studies of Bovine Ocular Squamous-Cell Carcinoma and Its Benign Precursor Lesions [1, 2, 3, 4]

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SUMMARY

The behavior of cells grown *in vitro* and derived from squamous carcinoma of the eye of cattle and its benign precursor lesions has been studied. Successful cultures have been obtained from 200 of 223 specimens (86%) of the different lesions. Cells from some of the lesions have been maintained for periods of more than 8 months and 173 serial passages. Cells of 15 percent of plaque, 12 percent of papilloma, and 26 percent of carcinoma specimens have shown characteristic changes after a minimum of five or more serial transfers. The changes consist of cytoplasmic vacuolization that may or may not be accompanied by cytoplasmic inclusion formation. The inclusions are eosinophilic and may occur without prior vacuolization of the cytoplasm. The changes occur in discrete foci, spread centrifugally, and may lead to destruction of the culture. None of these changes have been observed in cells of cultures derived from tissue of the limbal region of eyes of cattle apparently free of the disease. The possibility of these changes being due to a viral agent is discussed.—J. Nat. Cancer Inst. 26: 445–471, 1961.

BOVINE OCULAR squamous carcinoma, more commonly known as "cancer eye," has been recognized as a disease of certain breeds of cattle since the middle of the nineteenth century (*1*). Its causation has been attributed to many environmental factors (*2–9*). The work of Black (*10*) suggested, however, that environmental conditions were not as important in the etiology of the disease as at first considered. More recently, Anderson (*11, 12*) has shown that heredity is one of the major factors in the causation of the disease. Anderson (*13*) has also postulated that diet may play some part in the development of cancer eye. Wynne (*14*) has observed the marked tendency of the lesions that occur in this disease to undergo disappearance, reappearance, and a considerable degree of bilaterality. The observations of Russell *et al.* (*15*) sug-

[1] Received for publication August 15, 1960.

[2] This work was aided by grant C-1751 (C6) from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] The assistance of ranch owners and veterinarians in this study is gratefully acknowledged.

[4] This publication is No. XII of the "Cancer Eye" Study Section.

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gested that the disease may be similar to other animal tumors of known viral origin. Early attempts at transplantation of the bovine squamous-cell carcinoma by Loeb (*1*) failed. Similarly, Wynne *et al.* (*16*) failed in their attempts at transmission when they used extracts of fresh lesions. A small number of lesions that developed after such treatment could have been expected to occur by chance at the site of inoculation.

After these early studies and the report by Russell *et al.* (*15*) on the pathological anatomy of bovine ocular squamous-cell carcinoma, studies were undertaken to determine whether any characteristic changes could be detected in cells derived from cancer eye lesions after their growth *in vitro*. Russell *et al.* (*15*) observed a definite sequence in the appearance of lesions in this disease and described the three phases: plaque (epithelial hyperplasia), followed by papilloma, both benign lesions, after which carcinoma may occur. Many lesions are transitional between any two of the three stages already mentioned. The progression of the disease is not limited to the sequence plaque-papilloma-carcinoma (*15*). Any stage may "spontaneously" appear or disappear, though the disappearance of carcinoma is rare. Plaque may become carcinoma or papilloma, and alternatively it may regress completely; similarly papilloma may regress or proceed to carcinoma (*15*).

MATERIALS AND METHODS

Material used in the present study was collected in the field, usually during the spring or fall roundup of range cattle. Large numbers of both range and registered animals were made available for these investigations. Due to the exposed nature of the lesions, the ease of their contamination, and the high ambient temperatures in Texas and Oklahoma, a method was devised for the collection and transport of the material to the laboratory from a field trip of up to 7 days' duration. The method described allowed a high percentage of successful primary cultures. For the collection of lesions in the field, it was necessary to carry the equipment in as compact a manner as possible. We solved the problem by fitting out two ammunition boxes, 10½ × 19 × 8 inches outside dimensions, one for instruments, glassware, and solutions required during the collection of the lesions, and the other for the equipment and glassware used in processing the lesions for transport back to the laboratory. A small icebox of similar dimensions that contained crushed ice was used to maintain the various nutrient fluids and the processed lesions at a temperature of 4° C.

The collection of lesions was made in the following manner: The selected animal was caught and suitably restrained in a squeeze chute equipped with a head gate. The retro-ocular cavity was infiltrated with 30 to 50 ml of 2 percent procaine. After a short waiting period the eyeball could be pushed forward in the eye socket by digital pressure, which simplified the removal of the lesion. After removal the lesion was immedi-

ately placed in a sterile, 60 mm petri dish. The eyeball was then eased back in the socket by a simple lifting of the upper eyelid. Frequently, in these field operations, 50 or more cattle were operated on during a day and the provision of separate sterile equipment for each lesion was impossible. The field operations, therefore, were made with three sets of forceps, scissors, and petri dishes. Each set, after use on 1 animal, was wiped clean with a gauze square moistened with Tyrode's solution, flamed in an alcohol lamp, and allowed to cool before re-use. This method proved satisfactory, and contamination of cultures from bacterial sources was negligible. However, contamination, by molds and actinomycete-like organisms resistant to the antibiotics used, occasionally occurred. No satisfactory antifungal agent has yet been found suitable for these investigations.

During these field trips, material was also collected for future electron microscope studies. The specimens were therefore divided into three parts: one for histology, one for electron microscopy, and the remainder for tissue culture. The specimen for histology was placed in a small, screw-capped vial containing Bouin's fixative, and the piece for electron microscopy was immediately fixed in a mixture of equal parts of 1 percent osmic acid (*17*) and 1 percent potassium dichromate (*18*). The piece selected for tissue-culture study was placed in a small screw-capped vial containing Eagle's complete basal culture medium, with 20 percent calf serum, 200 μ/ml penicillin, 200 μg/ml streptomycin, and 200 μg/ml Erythrocin (supplied through the courtesy of Abbott Laboratories, North Chicago, Ill.).

The vial was then placed in a stainless-steel box compartmented and insulated with foamed polystyrene for storage in the icebox. After collection, the specimens for tissue culture were held at icebox temperature for 12 to 18 hours before preparation for transportation to the laboratory. This procedure gave bacteriologically cleaner specimens without impairing the viability of the tissues.

The method of preparation of the lesions that gave the highest percentage of successful primary outgrowths was as follows: Each lesion was removed from its vial with sterile forceps (the forceps were re-used for the next lesion after flaming). The lesion was placed in an individual petri dish and finely minced with a pair of curved iris scissors. Freshly prepared 0.2 percent Difco Bacto-Trypsin, in calcium and magnesium-free Hanks' salt solution, was added in sufficient quantity to wet thoroughly the minced-tissue fragments. Trypsinization was continued for 20 to 30 minutes at ambient temperature (78–98° F), with occasional agitation of the dish to ensure maximum action of the enzyme. After trypsinization the contents of the dish were transferred, with a fresh sterile capillary pipette for each specimen, to a screw-capped test tube containing 15 ml of complete medium. The tubes were then stored in the icebox until arrival at the laboratory. As already described (*19–21*), a high percentage of successful primary cellular outgrowths was obtained by the use of this technique.

After arrival at the laboratory, the tissue suspension was divided into suitable aliquots and inoculated into T-30 culture flasks. The flasks were incubated at 38° C and allowed to remain undisturbed for at least 4 days before the first microscopic examination. Cells derived from cancer eye lesions are slow to adhere to the glass, and appearance of any primary outgrowth may take as long as 30 days. The medium was routinely changed every 4 days, whether or not any change in the *p*H of the medium had occurred. The cells obtained from such tissues appear to be epithelioid, very thin, and therefore difficult to visualize by low-power, bright-field microscopy. We subcultured the cells by gently scraping them off the glass with a fish-tailed rubber policeman, for transfer to a fresh flask. Trypsinization was successful with some cultures, but generally the cells were found very resistant to trypsinization. Many cell lines adhered to the glass even after 1 hour of treatment with 1 percent trypsin at 38° C. To permit a more detailed study of the cells, wherever possible, subcultures were made into specially designed culture chambers (*22*, *23*) and cells were examined at high magnifications with phase objectives. The microscope system used was a Zeiss microscope, Type GFL, equipped with long-distance phase condenser, Type III Z/6. Neofluar phase objectives were used as they have a higher numerical aperture. Photomicrographs were made with a 35 mm camera attachment with a photo eyepiece #II having a built-in photocell. A Zeiss exposure meter was used to match the impedance of the photocell. Black and white photographs were taken with Kodak Panatomic X or Ilford Panchromatic F type films. Both films have a fine grain structure and permit enlargements of up to 8 times without noticeable grain. To obtain shorter exposure times and better contrast with fine grain structure, the films were developed in Ilford Microphen. Color pictures were taken on Kodak Ektachrome Type F.

RESULTS

Successful tissue cultures have been obtained from 200 of 223 specimens of different cancer eye lesions. The length of time in culture of cells derived from plaque, papilloma, and carcinoma lesions and from tissues of the limbal region of eyes from apparently disease-free cattle is shown in table 1.

Plaque

Phase microscopy of cells in tissue cultures derived from plaque lesions demonstrated cells which, by size and appearance, could be differentiated into two groups—one of larger size and epithelioid, the other of smaller size and more fibroblastic. Multinucleated cells and the occurrence of small, cytoplasmic vacuoles are common in such cultures (fig. 1). Well-marked striae that are tentatively classed as tonofibrils are often seen in cultures derived from plaque lesions (fig. 2). In the 2 cells shown at a higher magnification in figure 3, a more striking example of the same

TABLE 1.—Comparison of culture data for various cancer eye lesions

Type of lesion	Number of cultures examined	Number of cultures growing: Number of passages (months) Up to 1	1 to 4	4 to 8	Over 8	Cultures showing changes: Number	Percent
Plaque	89	50/2	28/19	8/13	3/49	13	14.6
Papilloma	49	23/1	18/18	5/29	3/51	6	12.0
Carcinoma	58	24/1	19/7	10/27	5/45	15	26.0
Normal	4	2/2	1/17	—	1/173	—	—

phenomenon can be seen. Cytoplasmic bridging is a frequent occurrence in such cultures and also occurs frequently in cultures derived from the other two types of lesions as well as in cells grown from normal eye tissue. Figure 4 shows an example of such bridging and early cytoplasmic inclusions. More clearly defined cytoplasmic inclusions are shown in the lower cell of figure 5 and are a later stage of inclusion development, after which the cells soon die and disintegrate. The process spreads centrifugally from such foci and results in the loss of the whole culture.

Papilloma

Cultures derived from papilloma lesions generally show cells with a remarkably uniform cytoplasm and nucleus (fig. 6). However, as in plaque lesions, larger cells are occasionally found. Vacuolization of the cytoplasm occurs less often than in the cells derived from plaque and carcinoma lesions. As shown in figures 7, 8, and 9, cells with multiple nuclei are occasionally seen in papilloma cultures, and cytoplasmic inclusions are not infrequent. In the cells derived from papilloma lesions, cytoplasmic vacuolization precedes the development of inclusions (*see* central cell, fig. 7). The inclusions, which are frequently encountered in cultures derived from cancer eye lesions, occur in the cytoplasm as dense, homogeneous masses and, when viewed by phase microscopy, may or may not be associated with cytoplasmic vacuolization (figs. 8 and 9). These inclusions stain deeply acidophilic with May-Grünwald-Giemsa stain and precede the type of cell death illustrated in figure 10.

Carcinoma

Cell cultures derived from ocular squamous-cell carcinoma lesions show a remarkable uniformity in the size of nuclei and in the distribution of chromatin. There is little variation in the size of cells in such cultures (fig. 11). Dense, homogeneous inclusions are found in the cytoplasm (fig. 12) and often precede the appearance of inclusions within cytoplasmic vacuoles. One of the multinucleated cells that contains both stages in the formation of cytoplasmic inclusions is shown in figures 13 and 14 (a composite picture made by joining pictures of adjacent cells in the

same culture). A high magnification of the nucleus and perinuclear region from a similar cell is shown in figure 15. This picture demonstrates in greater detail the variation in the appearance of cytoplasmic inclusions found in cultures of cancer eye lesions. It is possible that the inclusion in the juxtanuclear position could be a degenerated nucleus; however, at no time were 2 nuclei seen during routine daily examinations of this cell in the chamber culture.

Normal

Cells grown in culture, from tissue removed from eyes in which no gross or microscopic lesions were visible, do not show any changes associated with cultures derived from the three lesions of the disease. A low magnification picture of cells derived from the so-called normal eye tissue is shown in figure 16. As appears common for cultures from the three lesions of cancer eye, cultures derived from normal eye tissue taken from the limbal region show two main cell types: one larger and epithelioid, the other smaller. Two of the larger cells are seen at a slightly higher magnification in figure 17, which show the appearance of well-marked tonofibrils. Cytoplasmic bridging is well developed and common, as shown in figure 18. At no time has any of the changes described in the cells derived from plaque, papilloma, and carcinoma been seen in cells derived from the so-called normal eye tissue.

A study of stained preparations of cells grown *in vitro* and derived from the same types of cultures indicates that the inclusions are eosinophilic. Figure 20 shows a cell, derived from a plaque lesion, which evidences marked vacuolization between the two nuclei. Figure 21 reveals an early stage of inclusion formation within the two vacuoles adjacent to the nucleus. In cultures derived from papilloma, the cytoplasmic vacuolization is often more marked, as illustrated in figure 22. The inclusions that occur in the cytoplasm of papilloma cells are very similar to those seen in cells of plaque lesions but stain more deeply eosinophilic, as shown in figures 23 and 24. As seen in cells derived from carcinoma, the staining characteristics of the inclusions are comparable to those which occur in plaque lesions as indicated at low magnification in figures 25 and 26.

Cells derived from apparently normal eyes, even after 173 passages, have never shown any of these changes which occur in the cells from cancer eye lesions. As is clear in figure 19, cells from normal eye tissues demonstrate nuclear budding in one of the larger cells. This is a common finding in all cultures derived from normal and malignant eye tissues.

DISCUSSION

The findings of Russell *et al.* (*15*), who described the occurrence of intranuclear, spherical eosinophilic inclusions, and of Wynne (*16*), who demonstrated the bilaterality, appearance, disappearance, and reappear-

ance of cancer eye lesions, suggested a possible viral etiology or association of a viral agent with bovine ocular squamous carcinoma. Preliminary electron microscope studies by Dmochowski *et al.* (*24*) showed that the intranuclear inclusions were not viral in nature but nucleoli, abnormal in size and shape. These studies also demonstrated the presence of discrete particles in the cytoplasm of some cells that morphologically resembled viruses, and led to a tissue-culture study of cells derived from cancer eye lesions. The present investigation was initiated to study the cells under these conditions and to determine whether the presence of a viral agent would be revealed during growth of such cells *in vitro*.

This study suggests that an agent is carried by some of the cells grown in tissue culture and derived from the three lesions associated with the disease. Studies are in progress to determine whether cell-containing and cell-free extracts of pooled lesions of the same type will induce, in embryonic bovine kidney or corneoscleral cell cultures, changes similar to those observed in the cultures derived from the primary lesions and described in the present communication. Similar studies are being made with cell-containing and cell-free culture fluids, which have been stored at −70° C, and were derived from cancer eye lesion cultures with characteristic changes. These changes, nearly twice as frequent (26%) in cultures originating from carcinoma as from either plaque (14.6%) or papilloma (12%), are first noted as excessive vacuolization of the cytoplasm with an increase in density and granularity in the region surrounding the nucleus, and are followed by the appearance of inclusions that may or may not be within vacuoles in the cytoplasm. The inclusions stain deeply eosinophilic when fully developed, but may have a slight basophilic-eosinophilic reaction in the earlier stages of their formation. Such inclusions within a cell lead to its death and disintegration. The death of cells within a culture proceeds from distinct foci of a few affected cells in an otherwise apparently healthy culture. One or more culture flasks of a particular cell line will show similar changes at the same time. In view of this, the changes cannot be associated with nutritional defects or an unfavorable *p*H value of the culture fluid. Their appearance in a culture cannot be predicted on the basis of the clinical appearance of the original lesion. The age of the host and the age of the lesion do not appear to be associated with the occurrence of the changes observed in the cells derived from the various lesions grown *in vitro*.

In cell cultures derived from plaque lesions the changes appear most frequently during or after the fifth passage *in vitro*, irrespective of the total time in culture. Likewise, in cultures derived from papilloma lesions the changes occur most frequently after seven or more passages, whereas in carcinoma-cell cultures they appear later, between the fifteenth and 27th passages.

These studies suggest that a viral agent may be associated with the disease of bovine ocular squamous carcinoma and its benign precursor, lesions of plaque and papilloma, but that at present there is insufficient evidence to postulate a viral origin of this disease. Attempts are in

progress to determine whether the observed changes in cells derived from cancer eye lesions are due to a transmissible agent and its relationship, if any, to the disease.

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FIGURE 1.—Two types of cells with occasional small, cytoplasmic vacuoles. Ten-day-old chamber culture from fourth passage of a plaque lesion after a total of 59 days in culture. × 300

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FIGURE 2.—Two types of cells, some with well-marked striae. Seven-day-old chamber culture from 21st passage of a plaque lesion after a total of 98 days in culture. × 300

FIGURE 3.—Two cells shown at higher magnification with well-developed striae or tonofibrils. Eight-day-old chamber culture from 40th passage of a plaque lesion after a total of 210 days in culture. × 700

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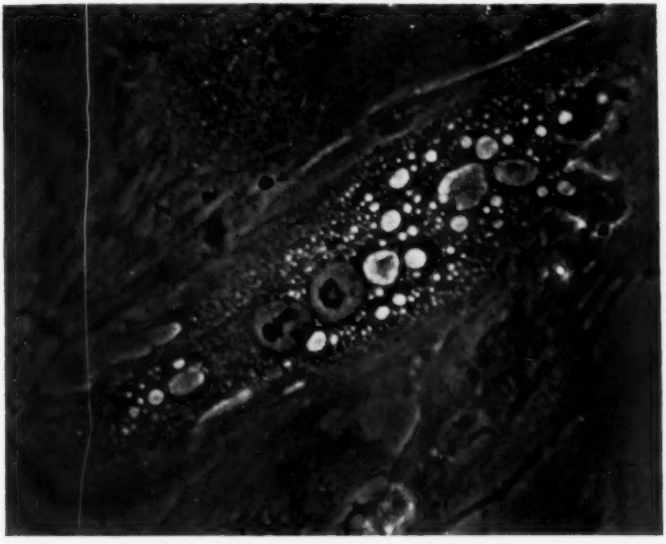
FIGURE 4.—Two cells showing well-developed cytoplasmic bridging and presence of early cytoplasmic inclusions. Nine-day-old chamber culture from 30th passage of a plaque lesion after a total of 75 days in culture. × 850

FIGURE 5.—Central cell shows a later stage in development of one type of cytoplasmic inclusion. Nine-day-old chamber culture from 40th passage of a plaque lesion after a total of 210 days in culture. × 700

FIGURE 6.—Cells of a culture showing regularity of cell size. Nine-day-old chamber culture from 22d passage of papilloma lesion after total of 180 days in culture. × 300



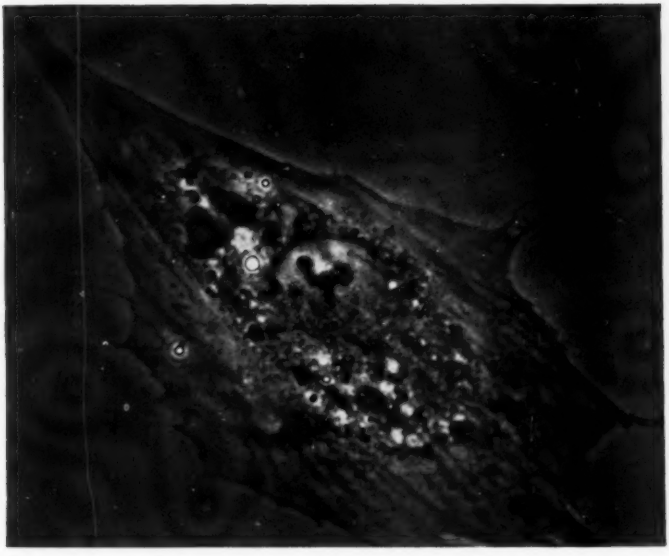


FIGURE 7.—Central cell shows 2 nuclei and excessive cytoplasmic vacuolization that usually precede formation of a cytoplasmic inclusion. Nine-day-old chamber culture from 22d passage of a papilloma lesion after 180 days in culture. × 700

FIGURE 8.—Cell showing numerous cytoplasmic inclusions that occur without prior vacuole formation. Five-day-old chamber culture of papilloma lesion from 37th passage after total of 240 days in culture. × 850

FIGURE 9.—*Upper* cell shows marked juxtanuclear vacuolization; *lower* cell shows presence of cytoplasmic inclusions. Cytoplasmic bridging is well marked. Five-day-old chamber culture from fourth passage of a papilloma lesion after total of 90 days in culture. × 850

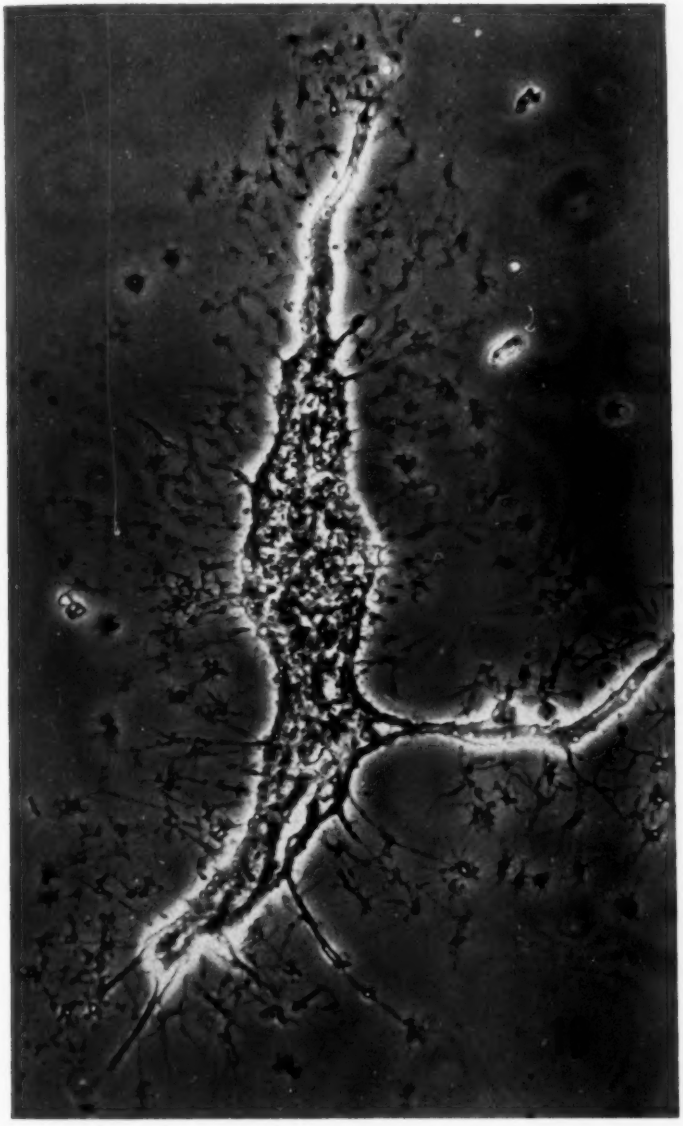


FIGURE 10.—Breakdown of a cell that commonly appears after cytoplasmic inclusions. Eight-day-old chamber culture from 22d passage of a papilloma lesion after total of 180 days in culture. × 700

FIGURE 11.—Regularity of cell size and presence of cytoplasmic inclusions in some of cells are shown. Six-day-old chamber culture from seventh passage of a carcinoma lesion after total of 60 days in culture. × 700

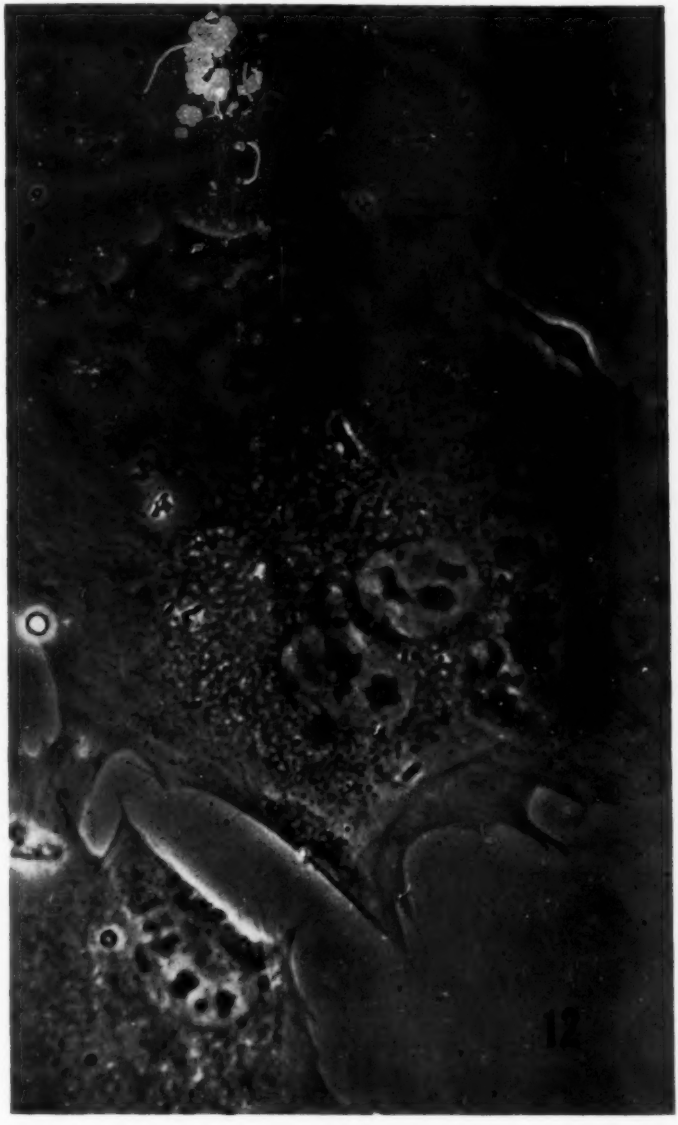


FIGURE 12.—Cell is shown with 2 nuclei and a cytoplasmic inclusion. Six-day-old chamber culture from ninth passage of a carcinoma lesion after total of 60 days in culture. × 750

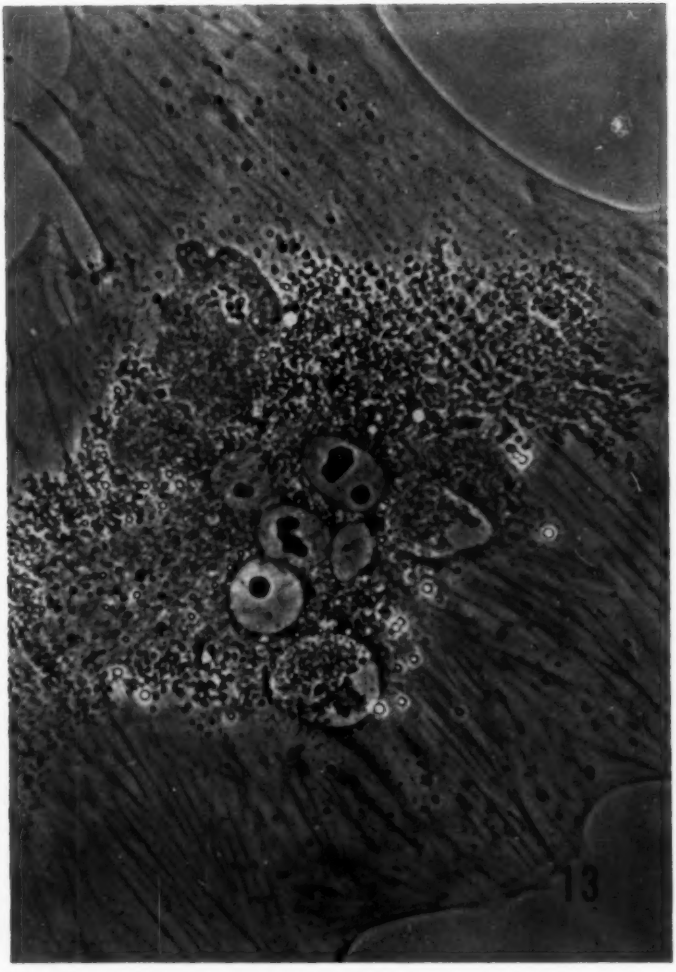


FIGURE 13.—Large multinucleated cell showing cytoplasmic inclusions. Eleven-day-old chamber culture from 31st passage of a carcinoma lesion after total of 225 days in culture. × 700

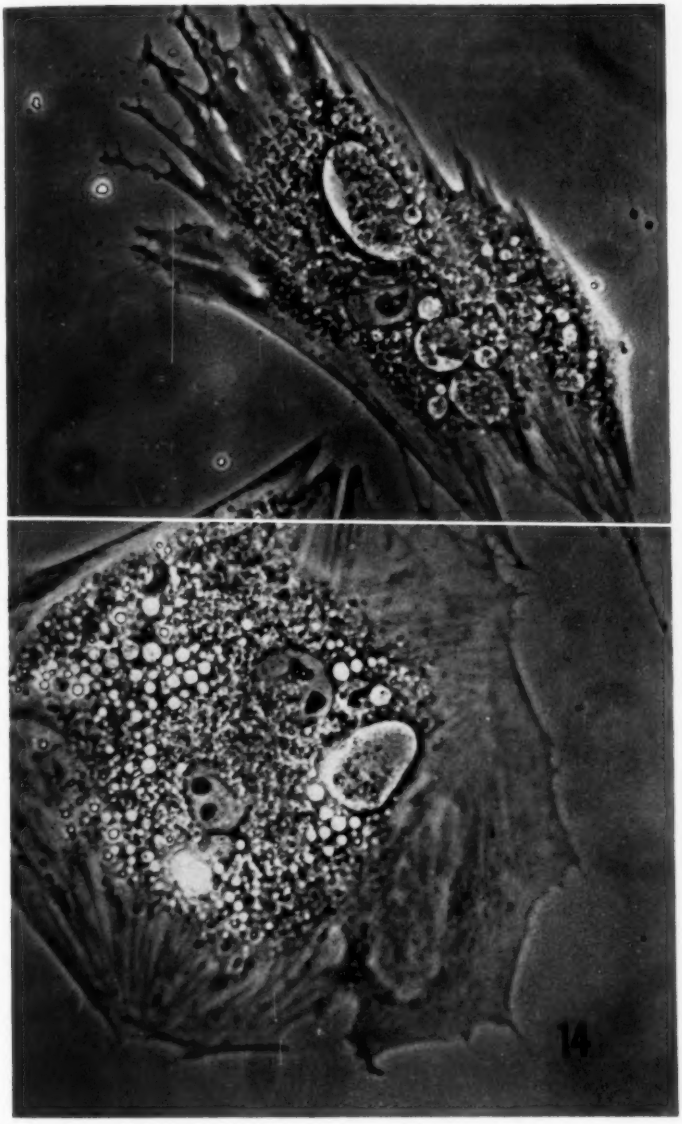


FIGURE 14.—Composite picture of 2 adjacent cells showing vacuolization and inclusions in cytoplasm. Eleven-day-old chamber culture of 31st passage from a carcinoma lesion after total of 225 days in culture. × 700

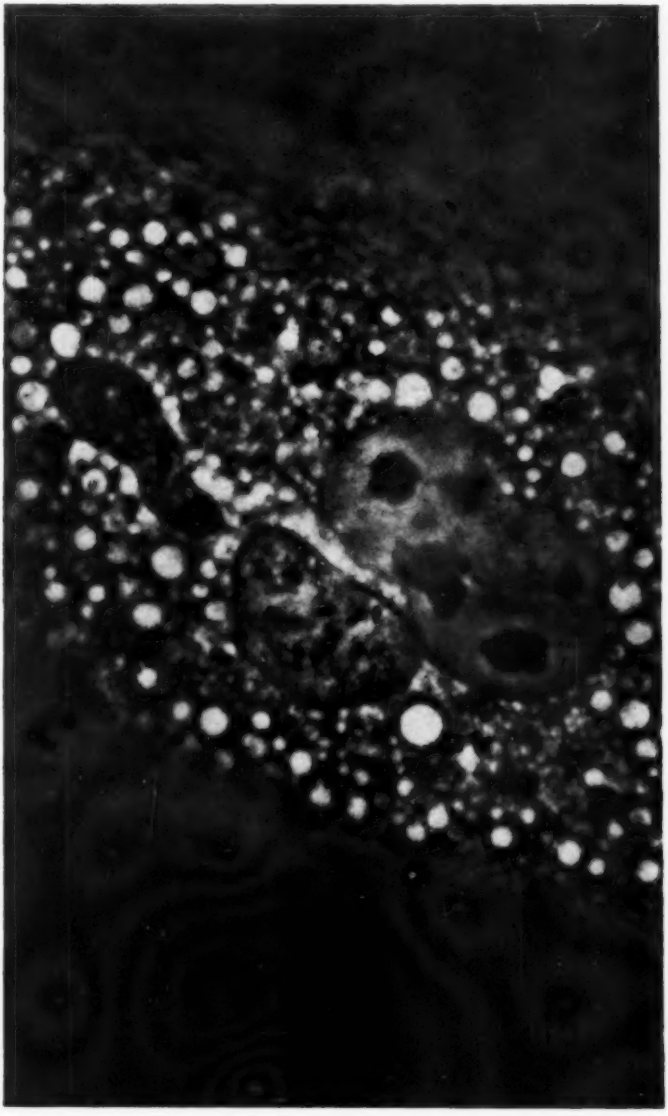


FIGURE 15.—Perinuclear zone of cell showing 2 cytoplasmic inclusions and many small vacuoles. Six-day-old chamber culture from 26th passage of a carcinoma lesion after total of 180 days in culture. × 2,800

FIGURE 16.—Low magnification view of culture derived from apparently normal eye tissue showing characteristic cells of large and small size. Ten-day-old chamber culture from seventh passage of normal eye tissue after total of 60 days in culture. × 300

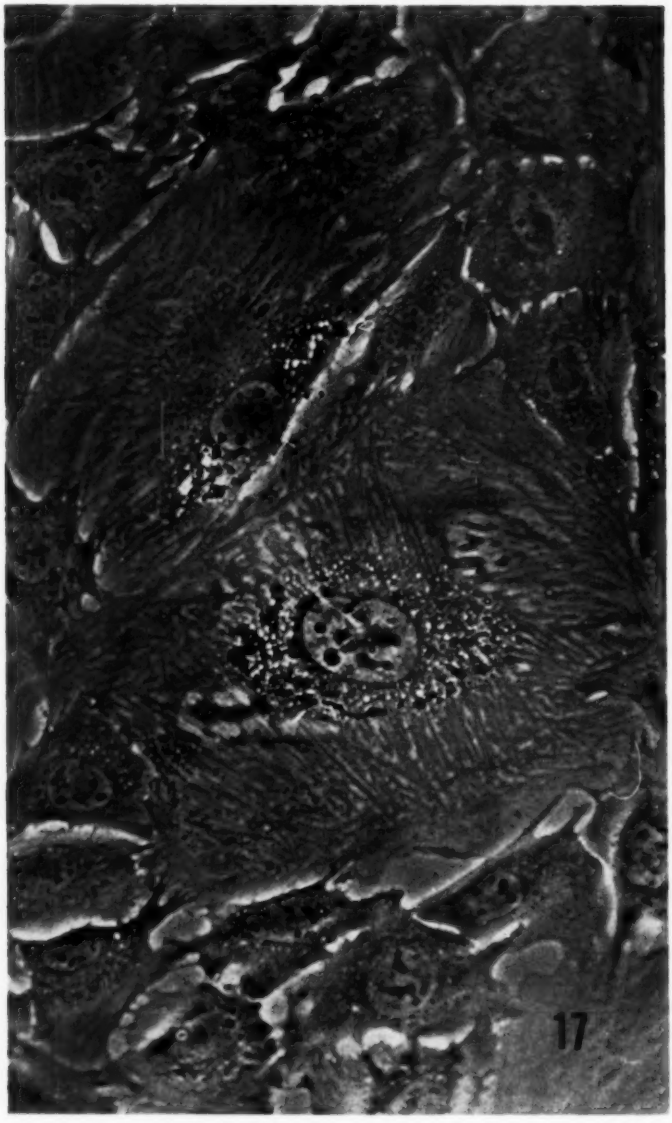


FIGURE 17.—Two of the larger cells showing well-marked striae or tonofibrils. Ten-day-old chamber culture from seventh passage of normal eye tissue after 60 days in culture. × 700

FIGURE 18.—Group of cells showing well-developed cytoplasmic bridging. Five-day-old chamber culture from 35th passage of normal eye tissue after total of 120 days in culture. × 700

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Cells shown in figures 19 to 26 have been fixed in 10 percent formalin buffered at *p*H 7.4 and stained with May-Grünwald-Giemsa.

FIGURE 19.—Cells of two sizes commonly found in cultures derived from corneal or corneoscleral tissues. In one of larger cells nuclear budding is demonstrated. Six-day-old chamber culture from twelfth passage of normal eye tissue after total of 97 days in culture. × 270

FIGURE 20.—Large cell showing vacuolization and cytoplasmic inclusions. Marked variation in staining of cytoplasm is probably due to variations in thickness. Five-day-old chamber culture from fourth passage of a plaque lesion after total of 68 days in culture. × 270

FIGURE 21.—Large cell showing 2 early inclusions that are stained weakly eosinophilic. Five-day-old chamber culture from fourth passage of a plaque lesion after total of 68 days in culture. × 1,000

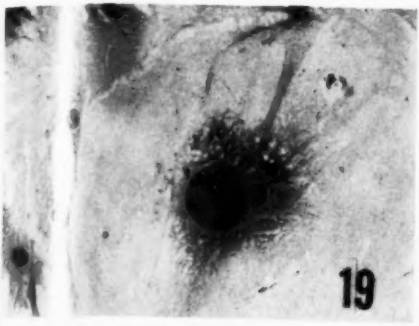
FIGURE 22.—Small area of culture showing cells with extensive cytoplasmic vacuolization. Several normal-appearing cells are also present. Nine-day-old chamber culture from 21st passage of a papilloma lesion after total of 97 days in culture. × 270

FIGURE 23.—Cell showing presence of well-developed eosinophilic cytoplasmic inclusion in perinuclear region and a juxtanuclear vacuole that appears to be an earlier stage of inclusion formation. Eleven-day-old chamber culture from 36th passage of a papilloma lesion after total of 223 days in culture. × 1,100

FIGURE 24.—Portion of cell showing deeply staining eosinophilic inclusion. Three-day-old chamber culture from 36th passage of a papilloma lesion after total of 223 days in culture. × 1,100

FIGURE 25.—Cells showing occasional small, cytoplasmic inclusions and increase in perinuclear granulation. Thirteen-day-old chamber culture from fourth passage of a carcinoma lesion after a total of 59 days in culture. × 270

FIGURE 26.—Cells showing variation in size common to cultures derived from cancer eye lesions. Somewhat later stage in the formation of a cytoplasmic inclusion is evident. Eleven-day-old chamber culture from fourth passage of a carcinoma lesion after total of 59 days in culture. × 270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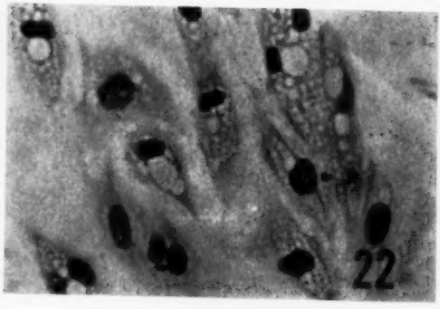
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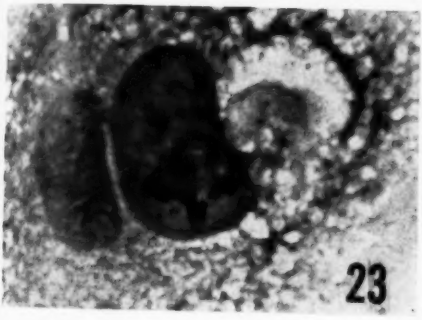
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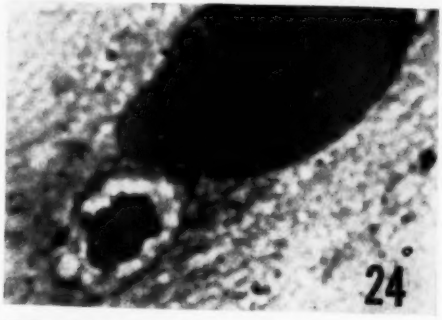
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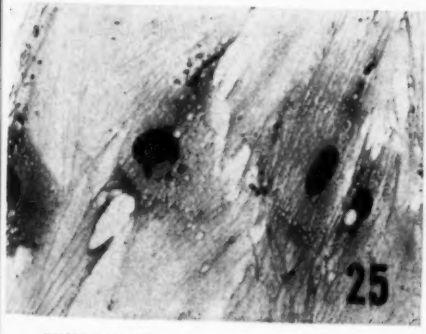
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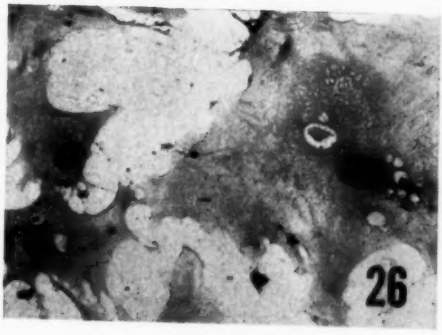
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Effects of Feeding Thiouracil on Thyroid Glands of Rats [1]

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SUMMARY

Inbred rats of the Fischer, A × C, and Marshall strains were fed thiouracil in a moderately low-iodine diet for various time intervals. The cells of the thyroid glands of these rats were arranged in several histologic patterns in addition to diffuse hyperplasia. These papillary and solid-cell patterns were localized in nodules or foci. There appeared to be strain differences in the frequency and type of focal change. Peculiar bodies with concentric laminations were observed frequently in the lumen of follicles in 2 strains. In many respects the results resembled those of Axelrad and Leblond obtained by feeding Wistar rats a low-iodine diet.—J. Nat. Cancer Inst. 26: 473-487, 1961.

THERE HAVE been many reports, summarized by Morris (*1*), of the production of tumors of the thyroid gland in mice and rats. Although transplantable tumors of the thyroid gland have been produced in inbred mice (*2*), there has been only limited success with transplantation of thyroid tumors in the rat (*3, 4*). It appeared likely that failure to obtain transplantable thyroid tumors in the rat was due to the use of noninbred animals. Attempts were therefore made to produce transplantable thyroid tumors in inbred rats by chronic feeding of thiouracil. This paper reports observations on the thyroid glands of 3 strains of rats fed thiouracil. Additional papers will report results of the transplantation of these thyroid glands. These papers will include information on histologic patterns and biochemical properties of the transplants.

MATERIALS AND METHODS

The 3 strains of inbred rats studied are listed in table 1. They were developed by Dunning and have been bred by the National Institutes of Health animal colony since 1950. Details of the history and properties of these rats were described recently by Billingham and Silvers (*5*).

[1] Received for publication August 25, 1960.

[2] I am grateful to Drs. Edward B. Price, Jr., and Leon Sokoloff for helpful suggestions, and to Mr. Donald F. Trainor for technical assistance.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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TABLE 1.—Inbred strains of rats studied

Strain	Color	When started	Generations inbred in 1951
Fischer 344	Albino	1920	54
A × C 9935	Brown	1926	44
Marshall 520	Albino	1920	56

Diet.—The animals were fed a pelleted diet of moderately low-iodine content. It contained 65.0 lbs. ground wheat, 23.3 lbs. skim-milk powder, 4.0 lbs. yeast, 8.8 lbs. corn oil, 1.4 lbs. analytical reagent-quality sodium chloride, 0.13 lb. iron citrate, 0.2 lb. shark-liver oil, and 0.08 lb. delsterol per 100 pounds of diet. The shark-liver oil and delsterol supplied vitamins A and D in amounts equal to 2 percent cod-liver oil in the diet. Thiouracil was incorporated into the pellets and fed to the Marshall 520 and the Fischer rats at a level of 0.25 percent of the diet, and to the A × C rats for the first 9 months at a level of 0.25 percent and later at a level of 0.50 percent.

At various time intervals after the rats were fed thiouracil, they were killed with ether, thyroids were weighed, and pieces were fixed in Zenker-formol. Some pieces of thyroids were implanted in rats fed thiouracil to test for transplantability. Histologic sections of thyroids were cut at 6 μ and stained routinely with hematoxylin and eosin. Some sections were stained by the periodic acid-Schiff method with hematoxylin counterstain, or by Snook's reticulum stain. Some additional stains were used in special studies and one such study will be mentioned.

RESULTS

Animals approximately 5 to 8 weeks of age at the start were fed thiouracil for varying time intervals (table 2). At autopsy the thyroids were generally enlarged and engorged with blood. The two lobes of the gland were usually approximately equal in size. In histologic sections of such glands, small nodules were occasionally observed that took up only a small fraction of the area of the section.

Histology.—The dominant pattern of almost all histologic sections was that of diffuse hyperplasia (fig. 1).

Colloid.—After 2 to 4 weeks of thiouracil feeding, colloid was largely depleted from all follicles. At 4 months, follicles or groups of follicles were occasionally filled with colloid, as described by Money and Rawson (*4*). In some strains, especially the Marshall rat, there were extensive deposits of colloid at 4 months. It was probable, however, that this was a consequence of an incomplete block of thyroid hormone formation. In the Marshall strain, and in the A × C also, with continued eating of the thiouracil-containing diet, food intake and therefore goitrogen intake were decreased. Variations in the amount of colloid present in thyroids

TABLE 2.—Summary of results

Histologic findings*

TABLE 2.—Summary of results

Strain	Time fed thiouracil (months)	Age at start (weeks)	Number started	Thyroid weight at necropsy (mg)	Number histology	Histologic findings*			
						Papillary nodules		Cellular nodules	Laminated bodies
						Columnar	Small cell		
Fischer 344	1	8	1 M†	56	1	0	0	0	—
	4	5	4 M	112 ± 5‡ (2)§	2	0	0	0	—
			4 F	89 ± 7 (2)	2	0	0	0	—
	6	8	1 M	120	1	1	1	0	—
	8	8	12 M	127 ± 27 (9)	9	1	0	1	1 ?
			9 F	87 ± 16 (8)	8	4	2	0	—
	9	5	1 M	160	1	1	0	0	—
			1 F	202	1	0	1	0	—
	12	8	1 F	—	1	1	1	0	—
	13	8	3 M	312 ± 32 (2)	3	3	0	0	—
A × C 9935	4	5	5 M	87 ± 6 (3)	3	0	0	0	—
			5 F	66 ± 7 (3)	3	0	0	0	—
	9	5	2 M	—	2	0	0	0	2
	24 to 30	5	8 M	—	2	0	0	0	2
			11 F	—	3	3	1	1	3
Marshall 520	1	8	1 F	64	1	0	0	0	—
	4	5	5 M	159 ± 29 (3)	3	0	0	0	—
			5 F	152 ± 6 (3)	4	0	0	0	—
	7	9	2 F	—	2	0	0	0	2
	8	8	12 M	209 ± 56 (10)	10	0	0	0	2
			10 F	147 ± 50 (7)	8	1 ?	0	0	8

*Number of thyroids with indicated pattern.

†M, F = male, female.

‡Mean ± standard deviation.

§Number weighed.

of experimental animals may have been due to variations in the daily intake of thiouracil among the animals. Because of the decrease in food intake, thiouracil was given temporarily in the drinking water to the Marshall rats, and the level of thiouracil in the diet of the A × C rats was raised to 0.5 percent.

Nodular Changes

Fischer 344 rats.—Focal or nodular changes were observed in the thyroids of some of the rats fed thiouracil for 6 months or more. The histologic pattern of the nodule was usually papillary (fig. 2), but there was possibly one example of a solid cellular pattern. The cells in papillary patterns were usually columnar with a hyperchromatic basally located nucleus (fig. 3). These cells resembled the type β_1 cells of Axelrad and Leblond (*6*).

In one papillary nodule some of the columnar cells, singly or in small groups, had pyknotic nuclei and especially densely staining cytoplasm (fig. 4). The significance of these cells is uncertain. In a few nodules the cells were more cuboidal (figs. 5 and 6).

In several cases two different types of cells occurred in the same follicle. A portion of the follicle was lined by cuboidal cells similar to those in diffuse hyperplasia. The remainder of the follicle was lined by columnar cells with hyperchromatic nuclei (figs. 7, 8, and 9).

In one papillary nodule composed of columnar cells, a projection into the lumen of a follicle appeared made up of a double row of small cells with densely staining nuclei (fig. 10). The base of the double row was at the apical end of columnar cells. No basement membrane was detected between the two rows.

A × C rats.—With one exception, nodules characterized by histologic patterns other than those of diffuse hyperplasia were observed only in females fed thiouracil for at least 2 years. Histologic patterns in the thyroids of such animals were much more complex than those in other rats fed thiouracil for a year or less. A section of a complex lobe (fig. 11) contained diffuse hyperplasia (peripherally) and, in addition, microfollicular and solid cellular patterns (figs. 12 and 13). In this gland, one lobe in which the histologic pattern was that of diffuse hyperplasia was much smaller than the other containing the complex pattern. In another gland there was a complex papillary area (fig. 14).

In one rat fed thiouracil for 9 months, a nodule was observed containing a fairly solid arrangement of columnar cells with periodic acid-Schiff positive cytoplasm (figs. 15 and 16). These cells were somewhat similar in appearance to the oncocytes of Hamperl (*7*). They differed, however, in that the cytoplasm of these cells was uniform and clear, whereas in the oncocytes the cytoplasm is granular.

Marshall 520 rats.—No nodules were observed in sections of the thyroids of the Marshall rat. In one follicle a small number of cells had hyperchromatic nuclei and looked as if they might be an early stage in formation of a papillary pattern.

The frequency of nodules with the characteristics described is listed in table 2. The number of nodules observed undoubtedly underestimates the number that occurs, for the data are based on sections through the gland obtained at a single random depth.

Laminated bodies in the lumen of follicles (figs. 17 and 18), similar to those described by Axelrad and Leblond, were observed many times in both A × C and Marshall rats, but rarely in the Fischer rat (table 2). Sometimes there was only one body in a follicle, but occasionally there were many. These bodies resemble the psammoma bodies described in human thyroid tumors by Klinck and Winship (*8*), except that they occurred within the lumen of follicles instead of between follicles. The appearance of these bodies was variable. In the Marshall rat the bodies were rarely hyperchromatic. At the suggestion of Dr. Leon Sokoloff, the following additional special strains were obtained on sections of a thyroid from an A × C rat (fig. 18): ninhydrin-Schiff for protein (*9*), periodic acid-Schiff (PAS), alizarin red S for calcium (*10*), and von Kóssa's silver nitrate method (*10*). The bodies stained positive for protein and polysaccharide, but with few weak exceptions were negative for calcium or phosphate. In the Marshall rat only a few laminated bodies were found in males, whereas they were frequent in approximately half the thyroids from females.

DISCUSSION

It is not possible to conclude that the nodular histologic changes observed in the thyroid glands of rats fed thiouracil are due to a direct action of thiouracil on the thyroid cell. The histologic changes are similar to those reported by Axelrad and Leblond (*6*) in Wistar rats fed a low-iodine diet and may therefore be a consequence of increased thyroid-stimulating hormone secretion.

The nodular changes that occur appear to have their origin in very limited regions of the gland. The present observations indicate that columnar cells with hyperchromatic nuclei were in some of the histologic sections localized to within a portion of a single follicle. Strain differences were observed in both the type and the frequency of nodules. It seems likely that the low incidence of nodules in the thyroids of the Marshall rat compared with the other strains is real. The large thyroid glands found in the Marshall rats at autopsy indicate that there was extensive stimulation by endogenous thyroid-stimulating hormone. Although some nodules in the A × C rats appeared to differ histologically from those in the Fischer rat, the differences may have been due partially to small sample size and partially to differences in the duration of thiouracil feeding.

Klinck and Winship (*8*) report that psammoma bodies isolated from thyroid tumors contain calcium phosphate. If the dense bodies of the type in figure 18 are, in fact, precursors of laminated bodies of the type

in figure 17, the present results suggest that the calcium phosphate is deposited on a preformed matrix of protein and polysaccharide.

Pieces of hyperplastic thyroid tissue from each strain were transplanted to rats of the same strain fed thiouracil. Implants took and grew in each strain. The implants after transplantation for several generations in the Fischer rat, grew in rats fed a diet to which no thiouracil was added. In later generations, implants grew in hypophysectomized rats. Studies were not continued in the A × C rat because the supply of rats was too limited. Studies in the Marshall rat were discontinued because growth of the implants was much slower than in the Fischer rat.

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PLATES

PLATE 72

FIGURE 1.—Primary hyperplasia. Thyroid gland from Fischer rat fed thiouracil for 8 months. Hematoxylin and eosin. × 167

FIGURE 2.—Papillary nodule. Fischer rat fed thiouracil for 8 months. Hematoxylin and eosin. × 80

FIGURE 3.—Columnar cells in papillary nodule, Fischer rat. Hematoxylin and eosin. × 465

FIGURE 4.—Groups of densely staining cells in area from papillary nodule. Fischer rat. Hematoxylin and eosin. × 950

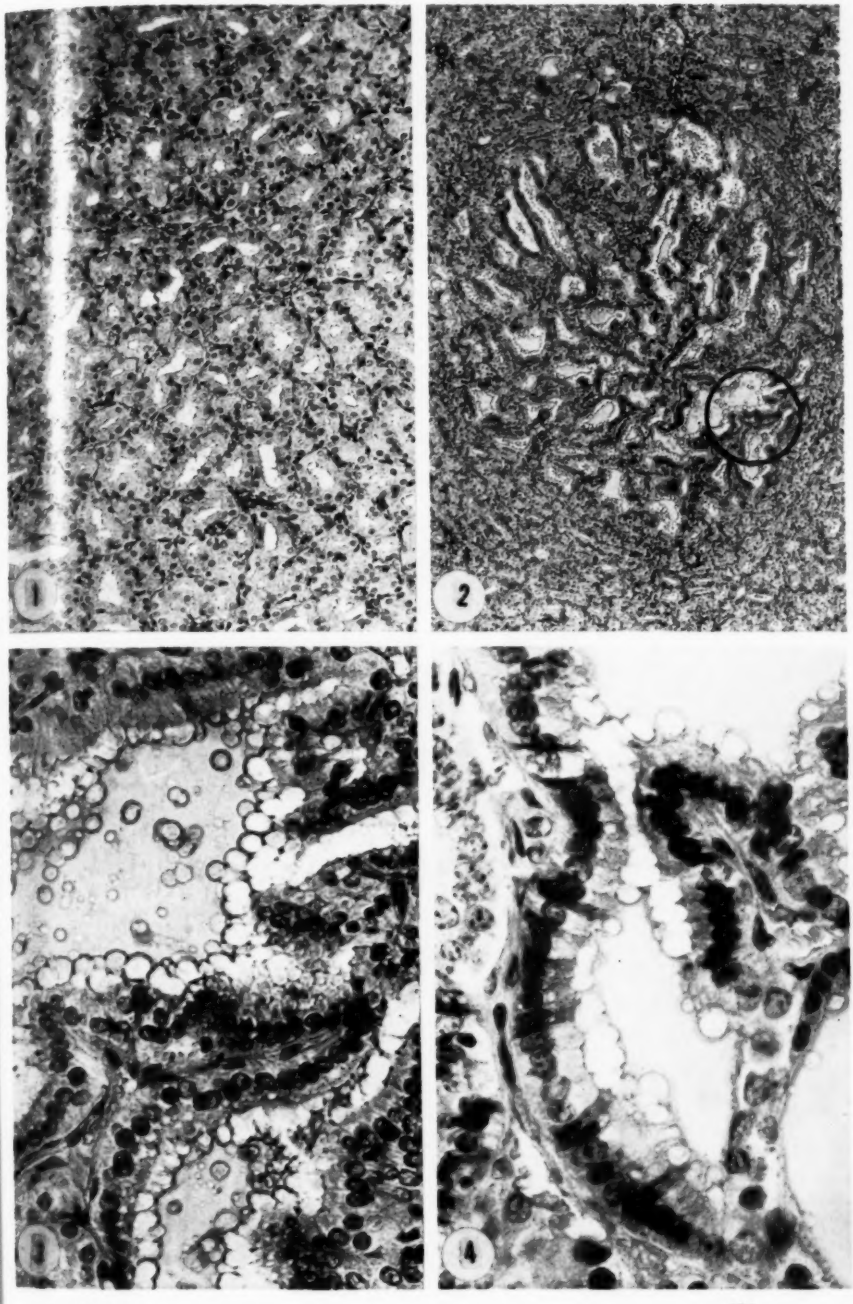

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PLATE 73

FIGURE 5.—Papillary nodule, more cuboidal type of cell. Fischer rat fed thiouracil for 8 months. Hematoxylin and eosin. × 167

FIGURE 6.—Area from nodule in figure 5. × 465

FIGURE 7.—Follicle composed of 2 cell types, cuboidal, characteristic of primary hyperplasia and columnar, characteristic of papillary nodules. Fischer rat fed thiouracil for 8 months. Hematoxylin and eosin. × 167

FIGURE 8.—Follicle composed of 2 cell types as in figure 7. Fischer rat fed thiouracil for 9 months. Hematoxylin and eosin. × 285

FIGURE 9.—Follicle composed of 2 cell types as in figure 7. Fischer rat fed thiouracil for 8 months. Hematoxylin and eosin. × 335

FIGURE 10.—Projection of small cells with hyperchromatic nuclei into lumen of follicle lined by columnar cells. Fischer rat fed thiouracil for 6 months. Hematoxylin and eosin. × 285

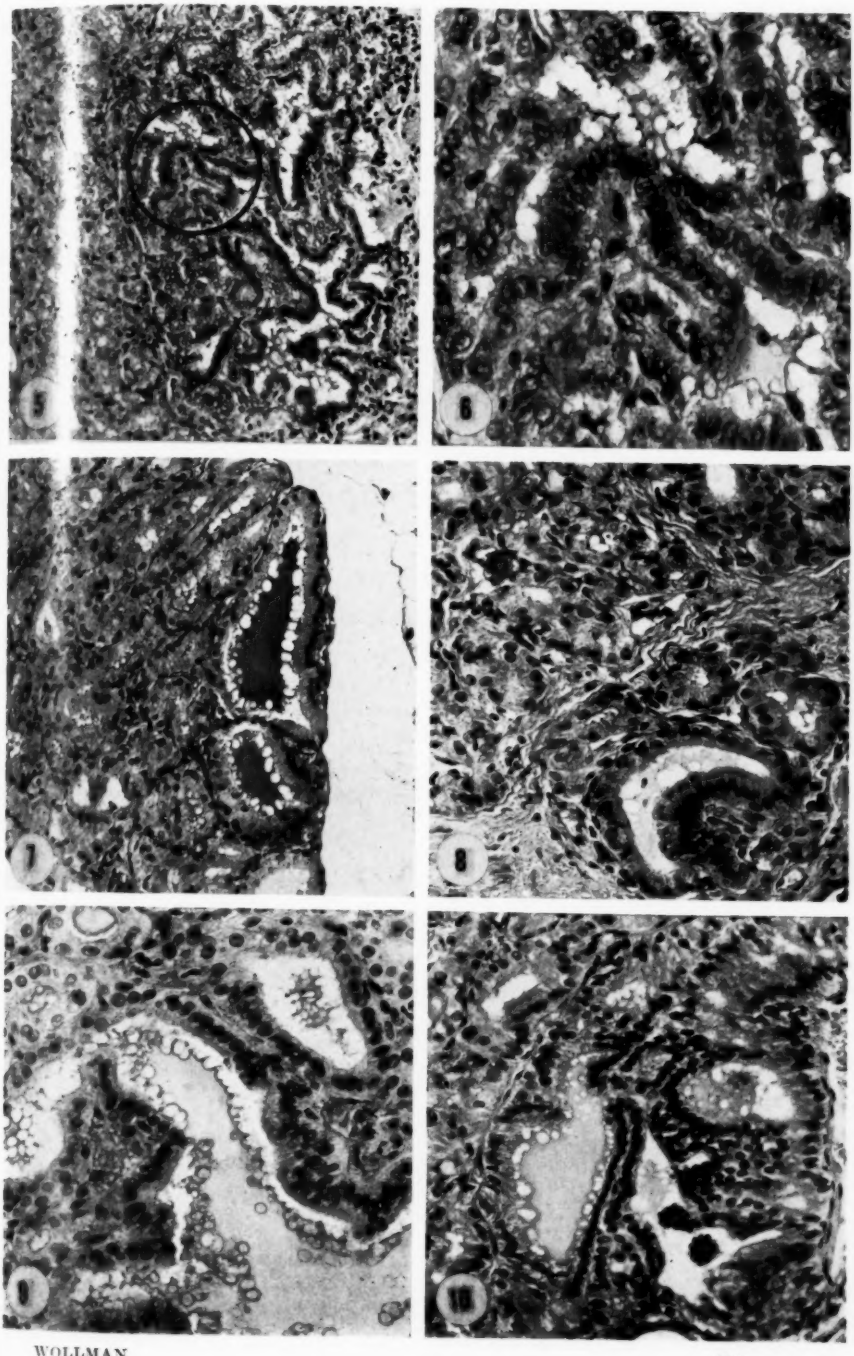
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PLATE 74

FIGURE 11.—Lobe of thyroid gland of A × C rat fed thiouracil for 2 years. Hematoxylin and eosin. × 9

FIGURE 12.—Detail of upper area in figure 11. × 167

FIGURE 13.—Detail of lower area in figure 11. × 167

FIGURE 14.—Detail of papillary area from thyroid gland of a female A × C rat fed thiouracil. Hematoxylin and eosin. × 167

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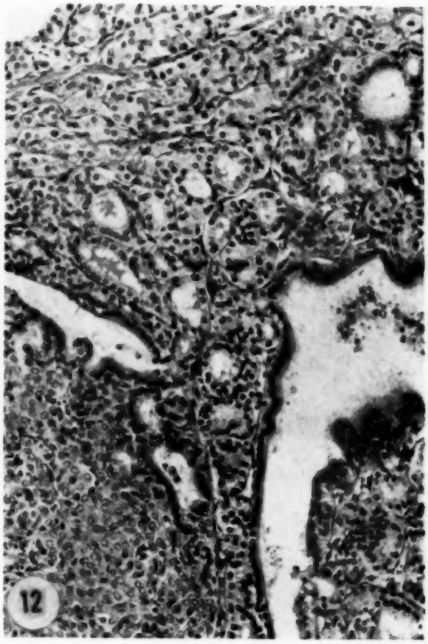
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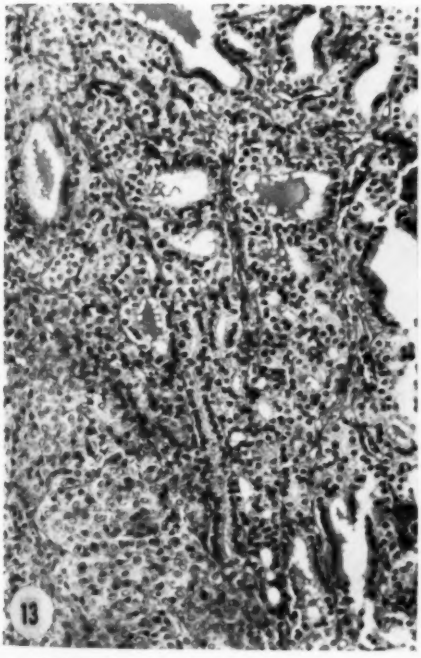
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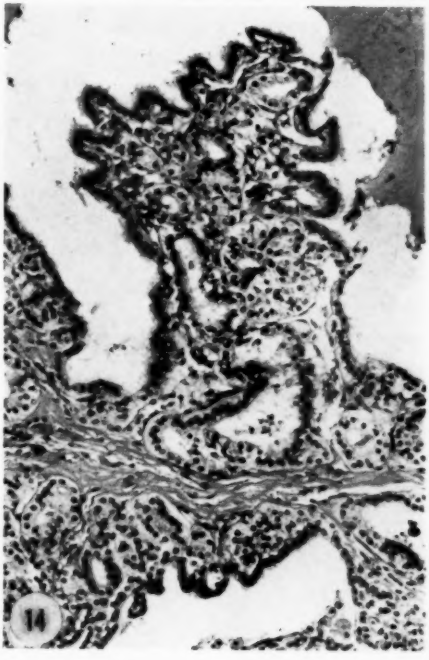
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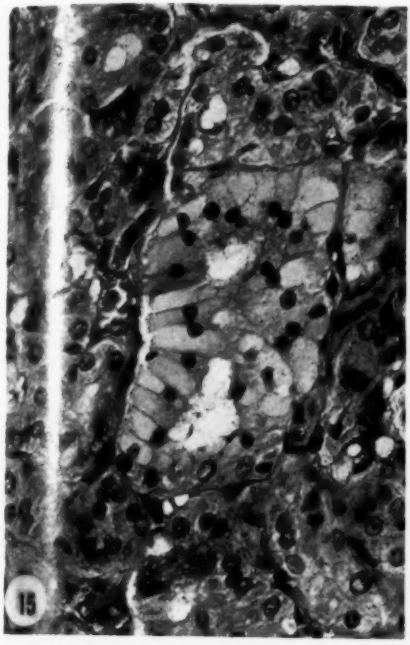
PLATE 75

FIGURE 15.—Large cell area in thyroid gland of A × C rat fed thiouracil for 9 months. Hematoxylin and eosin. × 465

FIGURE 16.—PAS-positive material present in large cells from section neighboring that illustrated in figure 15. Periodic acid-Schiff and hematoxylin. × 465

FIGURE 17.—Dense bodies with concentric laminations found in lumen of follicles of thyroid transplant in A × C rat fed thiouracil. Hematoxylin and eosin. × 465

FIGURE 18.—Dense bodies, some with laminations, found in lumen of thyroid follicles from A × C rat fed thiouracil for 9 months. Hematoxylin and eosin. × 465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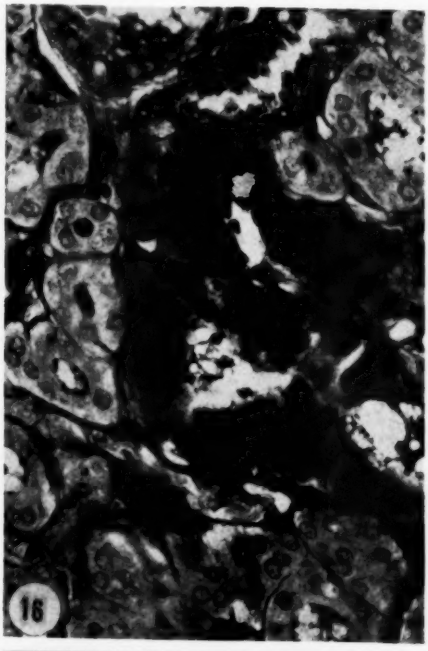
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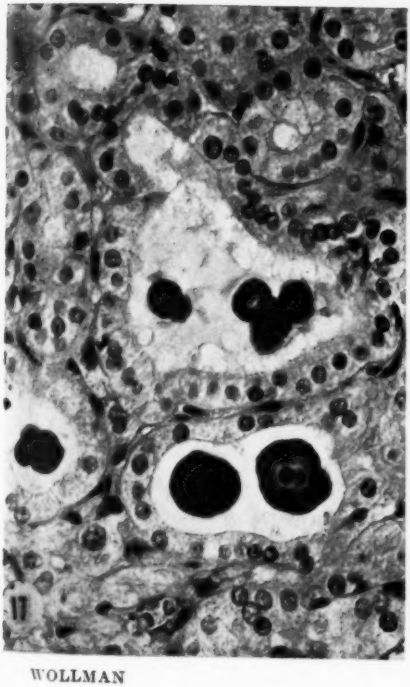
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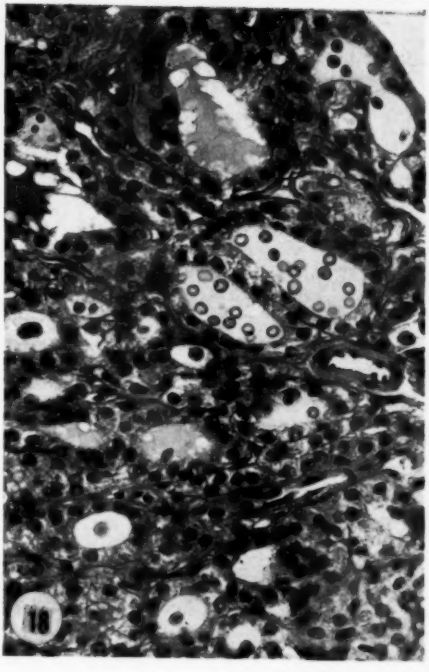
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Survival of Patients With Cancer of the Large Intestine and Rectum, Connecticut, 1935–54 [1, 2]

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SUMMARY

We are presenting a detailed 15-year survival analysis by stage, age, calendar period of diagnosis, and type of treatment for 7,128 patients with cancer of the large intestine and 5,082 patients with cancer of the rectum diagnosed in the State of Connecticut between 1935 and 1954. The data are unique in that they describe the experience of virtually all patients with these diseases in a community rather than that of patients admitted to an individual treatment center. Observed survival is measured relative to expected survival in the general population. For cancer of the large intestine and rectum, 5-year survivors experienced considerably higher mortality than persons of similar age in the general population. Ten-year survivors experienced approximately normal survival between the 10th and 15th years after diagnosis. Five-year relative survival rates improved dramatically from 1935–44 to the post-World War II period 1945–54: large intestine: males, 19 to 32 percent; females, 23 to 39; rectum: males, 15 to 30; females, 23 to 32. Improvement was noted for patients with localized disease and with regional spread. The relative number of untreated patients decreased from 1 in 4 to 1 in 6 for cancer of the large intestine, and from 1 in 5 to 1 in 7 for cancer of the rectum. More frequent use of surgery and increase in length of survival of the surgically treated patients contributed to the over-all improvement. Most of the increase in the 5-year relative survival rate for the surgically treated patients resulted from increase in the 1-year survival rate, which suggests that reduction of operative mortality was an important factor. Women tended to have a more favorable outlook than men, but for cancer of the rectum this sex difference disappeared during the second period of diagnosis, 1945–54.—J. Nat. Cancer Inst. 26: 489–510, 1961.

THIS IS the fifth in a series of reports on the survival experience of patients diagnosed with cancer in Connecticut hospitals during the 20-year period 1935–54. The first report dealt with cancer of the breast and included a discussion of the statistical method used in the analysis (*1*).

[1] Received for publication August 25, 1960.

[2] This study was made possible, in part, by financial assistance from the National Cancer Institute, National Institutes of Health, Public Health Service. The gathering of the basic data was partially supported by grant 9437; machine processing was supported by contract SA-43-ph-1903.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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The subsequent reports were on cancer of the uterus, ovary, and stomach (*2–4*). The data are based on information routinely submitted by 36 hospitals to the Tumor Registry operated by the Connecticut State Department of Health, the history of which has been described by Griswold and Cutler (*5*) and Greenberg (*6*). An available electronic computer at the National Institutes of Health made this analysis possible.[4]

The data from the Connecticut Registry are unique in that they provide information on virtually all known cases of cancer in a community, rather than on patients admitted to an individual treatment center. Whereas patients seen at individual treatment centers may be selected with respect to socioeconomic status, stage of disease, or general clinical condition, the data in this report describe the survival experience of *average* patients with cancer of the large intestine and rectum.

We are presenting information on 7,128 cases of cancer of the large intestine (International Statistical Classification No. 153) and 5,082 cases of cancer of the rectum (International Statistical Classification No. 154). Because this is a survival study, we excluded the very substantial number of cases [5] which were first diagnosed at autopsy or for which the death certificate was the only source of information. The percentage of these cases decreased markedly during the 20 years of study. We also excluded cases with a known prior history of cancer and 36 cases of unknown age.

Seventy-seven percent of the cases of cancer of the large intestine and 80 percent of the cases of cancer of the rectum were microscopically confirmed. We included the 1,659 clinically diagnosed cases of cancer of the large intestine and the 1,025 clinically diagnosed cases of cancer of the rectum in this analysis because we felt it desirable to evaluate the survival experience of all persons in the Connecticut community that were diagnosed with cancer of these sites. Also, the survival rates for clinically diagnosed cancer cases are markedly lower than for microscopically confirmed cases (*7*). This suggests that biopsy was probably considered either medically inadvisable or unnecessary for a large number of the clinically diagnosed cases. We have little doubt that many of the clinically diagnosed cases were in fact cancer, though the classification by primary site and stage is less certain than in the microscopically confirmed cases. In any event, including the clinically diagnosed cases depresses rather than increases the survival rates. Thus, the relative 5-year survival rate for the total population of male patients with cancer of the large intestine diagnosed 1935–44 was 19 percent, in contrast with a rate of 27 percent for the microscopically confirmed cases (table 5). The corresponding percentages for cancer of the rectum are 12 and 16 (table 13).

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[4] We are indebted to Mr. Elliot Cramer and to Mr. Michael C. Altschuler who programmed and executed the computations.

[5] Cancer of the large intestine, 2,585 cases; cancer of the rectum, 1,173 cases.

CANCER OF THE LARGE INTESTINE

Age and Sex

Of the 7,128 cancers of the large intestine (excluding the rectum), 3,231 were in men and 3,897 in women. To provide a basis for evaluating changes over time, we divided the patients into two cohorts according to calendar period of diagnosis: 1935–44 (2,458 patients) and 1945–54 (4,670 patients).

The age and sex distribution of the patients with cancer of the large intestine is shown in table 1. Nine out of 10 patients were 45 or older, and the male patients were, on the average, 2 years older than the female patients. The increase in age at diagnosis from the first calendar period, 1935–44, to the second, 1945–54, is noteworthy: The median age increased from 62 to 65, which reflected the increase in age of the Connecticut population. The 20 percent excess of female cases over male cases is typical for cancer of the large intestine (*8*, *9*).

TABLE 1.—Percentage age distribution, by sex and calendar period of diagnosis: patients with cancer of the large intestine diagnosed in Connecticut, 1935–54

	Both sexes		Male		Female	
	1935–44	1945–54	1935–44	1945–54	1935–44	1945–54
Number of patients	2, 458	4, 670	1, 167	2, 064	1, 291	2, 606
Percent:						
All ages*	100	100	100	100	100	100
Under 45	12	9	10	6	14	10
45–54	18	13	16	11	19	15
55–64	28	27	29	29	27	26
65–74	30	32	32	36	28	29
75+	13	18	14	18	12	19
Median age	62	65	63	66	61	64
Mean age	61	64	62	65	61	63

*Percentages may not total 100 because of rounding.

Survival Experience

Table 2 shows that the follow-up information is fairly complete. Only 198 patients were lost during the first 5 years of follow-up. Sixty-seven of the 925 patients who were known to have survived the first 5 years were lost between the 5th and 10th years. Of the patients diagnosed in 1935–44, 17 of the 267 known to be alive at the beginning of the 11th year were lost before the end of the 15th year. Subsequent to date of last contact, the lost patients were assumed to experience a survival pattern similar to that for the patients remaining under follow-up. This is in accordance with an earlier recommendation (*10*). The relatively small number of lost patients were distributed among the various subclasses (age, stage, treatment), so that it is unlikely that a large bias has been introduced into the computed survival rates.

Text-figure 1 shows the survival experience in 15 years of follow-up of the 1,167 male and 1,291 female patients with cancer of the large intestine

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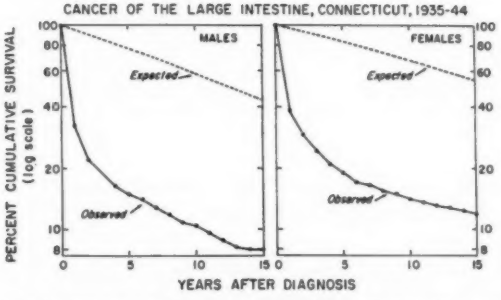
TABLE 2.—Follow-up status of patients with cancer of the large intestine diagnosed in Connecticut, 1935–54, by 5-year follow-up interval and calendar period of diagnosis

Calendar period of diagnosis and follow-up interval	Alive at beginning of interval	Died during interval	Lost to follow-up during interval	Withdrawn alive during interval*
Patients diagnosed 1935–44				
0–5 years	2, 458	2, 021	34	—
5–10 years	403	106	30	—
10–15 years	267	35	17	143
Patients diagnosed 1945–54				
0–5 years	4, 670	2, 816	164	1, 168
5–10 years	522	91	37	394

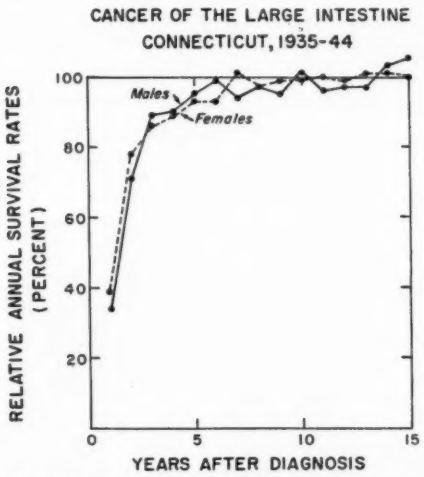
*Alive on closing date of study, December 31, 1954.



TEXT-FIGURE 1.—Observed and expected survival rates for 15 years of follow-up: patients with cancer of the large intestine diagnosed in Connecticut, 1935–44.

diagnosed 1935–44. The logarithmic scale used for plotting the percentage of survivors at the end of successive follow-up years permits visual evaluation of the rate of depletion (mortality). The very steep slope of the initial part of the curve both for males and females tells us that the mortality was very high: Only 1 of 3 patients survived the 1st year, and 1 of 6 survived the first 5 years. For comparison, we are showing the expected survival experience for a group of persons in the general population with an age distribution similar to that of the patients. Visually, the observed and expected slopes are roughly parallel after 5 years.

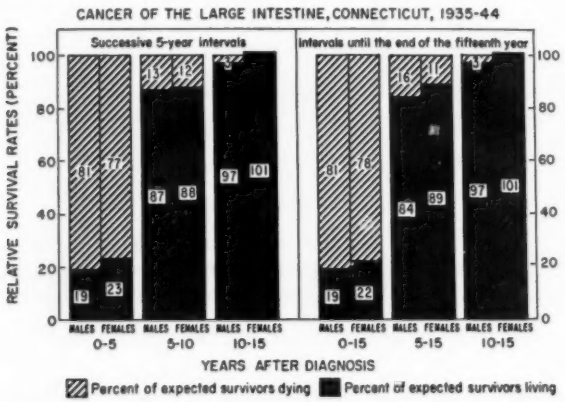
The annual relative survival rates shown in text-figure 2 provide a more precise measure of the relationship between the observed and expected curves of text-figure 1. The relative survival rate is the ratio of the observed to the expected survival rate. A relative rate of 100 percent means that during a specific follow-up year the mortality rates in the patient group and in the general population were equal. A relative rate of less than 100 percent means that mortality in the patient group was greater than expected on the basis of general population experience. The attainment and maintenance of annual survival rates in the patient group equal to that in the general population would indicate that excess mortality had been eliminated.



TEXT-FIGURE 2.—Relative annual survival rates for 15 years of follow-up: patients with cancer of the large intestine diagnosed in Connecticut, 1935–44.

From text-figure 2 we see that the annual relative survival rates for both males and females increase sharply from 1-year values of less than 40 to over 90 percent from the 5th year on. The 1st-year value of 40 percent is double the corresponding value for cancer of the stomach (*4*), but only about half that for cancer of the female reproductive system (*1–3*). Between the 10th and 15th years the values fluctuate around 100. It thus appears that the patients who survived 5 years were still subject to some excess mortality, but that those who survived 10 years were dying at a rate approximately equal to that of persons in the general population. We may confirm this by inspecting the *left panel* of text-figure 3, which summarizes the survival experience for successive 5-year intervals in terms of the relative survival rate. Only one fifth of the expected survivors survived the first 5 years after diagnosis. Of the patients alive at the end of the 5th year, seven eighths of the expected survivors lived until the end of the 10th year, and of the patients alive at the end of the 10th year, nearly all of the expected survivors lived until the end of the 15th year. It appears, then, that persons who survived cancer of the large intestine for 5 years were not yet experiencing normal mortality: about 1 out of 8 expected survivors died between the 5th and 15th years of follow-up (see *right panel* of text-fig. 3). However, the relative survival rate for the small proportion of persons (about 13%) who survived for 10 years is approximately 100 percent for the subsequent 5 years. (The computed relative survival rate for females for the 10- to 15-year interval is 101 percent, but this rate is not significantly greater than the rate of 97 for males.) The scanty data available for the 15- to 20-year interval (not shown here) suggest that the relative survival rate for that duration may also be approximately 100. It appears, then, that patients with cancer of the large intestine who survive for 10 years may be experiencing

normal survival thereafter. This is in sharp contrast to our findings for cancer of the female reproductive system (*1–3*), in which we observed excess mortality among those patients who survived the first 10 years. It must be remembered that patients with cancer of the large intestine who survived 10 to 15 years were diagnosed during 1935–44. Whether or not patients currently treated will have a similar long-term survival experience will not be known before 1975.



TEXT-FIGURE 3.—Relative survival rates for specified follow-up intervals: patients with cancer of the large intestine diagnosed in Connecticut, 1935–44.

Variation by Sex

Table 3 shows that women with cancer of the large intestine have a somewhat better survival experience than men. For the cases diagnosed 1935–44, the differences are not very large, *i. e.*, the 5-year relative survival rate is 19 for males and 23 for females, and the corresponding rates for the 5- to 10-year interval are 87 and 88, respectively. For the cases diagnosed 1945–54, the differences are larger: The 5-year relative survival rate is 32 for males and 39 for females.

Secular Trend

We are evaluating the change in rate of survival over time in terms of the *crude* and *relative* survival rate. The relative survival rate corrects for any decrease in deaths from other causes during the second period resulting from the lowering of general mortality in recent years, and also corrects for any increase in deaths from other causes resulting from the shift in the age composition of the patients. Table 3 shows that the differences between crude and relative survival rates are small over a 1-year interval, but that over intervals of 5 or more years normal mortality is substantial in a population whose average age is in the sixties.

There was a dramatic improvement in survival among patients diagnosed during the second period, 1945–54, over those diagnosed during

TABLE 3.—Survival experience, by sex: patients with cancer of the large intestine diagnosed in Connecticut, 1935–44 and 1945–54*

	Males			Females		
		Relative			Relative	
	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error
1935–44						
0–1	32	34	1.4	38	39	1.4
0–5	15	19	1.4	19	23	1.3
0–10	10	18	1.6	14	21	1.4
0–15	8	19	2.0	12	22	1.8
1–5	46	56	3.4	50	59	2.6
5–10	70	87	4.5	75	88	3.4
10–15	*77*	*97*	6.1	84	101	4.6
1945–54						
0–1	52	55	1.2	57	59	1.0
0–5	25	32	1.5	33	39	1.3
0–10	14	24	4.0	20	29	3.6
1–5	48	58	2.4	57	66	1.9
5–10	n.r.	n.r.		*60*	*72*	8.6

*Rates are expressed as percents. Rates in italics have standard errors between 5.0 and 9.9. Rates with standard errors of 10.0 or larger are not shown (n.r. = not reliable).

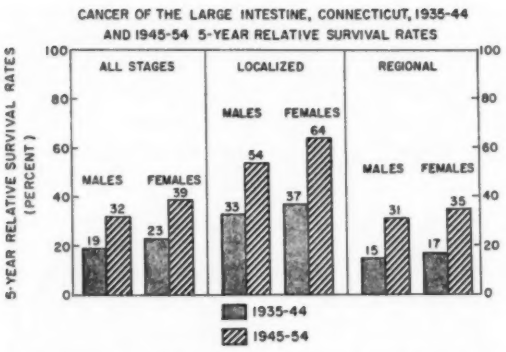
1935–44 (table 3 and text-fig. 4). The relative 5-year survival rate increased from 19 to 32 for men, and from 23 to 39 for women. This is a relative improvement of nearly 70 percent, a notable achievement. Interestingly, virtually all of this gain was accomplished during the 1st year of follow-up. The relative 1-year survival rates increased from 34 to 55 in men, and from 39 to 59 in women. The increase in relative survival rates for the 1- to 5-year interval was much smaller: from 56 to 58 in men and from 59 to 66 in women.

Variation by Stage of Disease

The classification according to stage of disease is based on all available information at discharge from the hospital. The following definitions were used:

1. Localized—cancer is confined to the site of origin.
2. Regional spread—cancer has passed the bounds of the site of origin; furthest spread thought to be limited to neighboring organs or tissues, or to regional lymph nodes.
3. Distant or remote spread—cancer has spread to organs or tissues beyond those immediately draining or neighboring the site of origin.

Table 4 shows the distribution of cases and table 5 and text-figure 4 the survival experience by stage of disease. There was no sex difference in stage distribution; therefore we combined the 2 sexes in table 4. Stage of disease was, of course, a very important factor in prognosis. Five-year survival for "localized" cases was approximately double that for "regional" cases. For patients with both localized and regional spread of disease survival improved greatly from the first to the second period. Thus, for men the 5-year relative rate increased from 33 to 54 for "localized" cases,



TEXT-FIGURE 4.—Comparison of 5-year relative survival rates: patients with cancer of the large intestine diagnosed in Connecticut, 1945–54.

and from 15 to 31 for "regional" cases. It will be noted that the percent of cases classified as "localized" decreased from the first to the second period. This, however, is not necessarily a bona fide change in stage composition. A relatively greater number of patients were operated on in the second period, and it is likely that metastases were discovered during the operation that would otherwise not have been noted.

TABLE 4.—Stage distribution by microscopic confirmation of diagnosis: patients with cancer of the large intestine diagnosed in Connecticut, 1935–44 and 1945–54 (includes 21 patients of unknown age who were excluded from survival computations)

	All stages	Localized	Regional	Distant	Stage unknown
1935–44					
Confirmed and not confirmed, combined					
Number of patients	2, 465	1, 131	608	511	215
Percent	100	46	25	21	9
Confirmed only					
Number of patients	1, 600	777	445	320	58
Percent	100	49	28	20	4
1945–54					
Confirmed and not confirmed, combined					
Number of patients	4, 684	1, 895	1, 477	900	412
Percent	100	40	32	19	9
Confirmed only					
Number of patients	3, 882	1, 704	1, 343	681	154
Percent	100	44	35	18	4

Treatment

The patients were classified into three broad categories according to the treatment they received during the first course of medical care: surgery,

TABLE 5.—Crude and relative survival rates by stage of disease and microscopic confirmation of diagnosis: patients with cancer of the large intestine diagnosed in Connecticut, 1935–44 and 1945–54

Sex, confirmation, follow-up interval in years, and period of diagnosis	All stages*		Localized		Regional	
	Crude survival rate	Relative survival rate	Crude survival rate	Relative survival rate	Crude survival rate	Relative survival rate
Males						
Confirmed and not confirmed, combined						
0–1						
1935–44	32	34	42	44	38	39
1945–54	52	55	71	75	59	61
0–5						
1935–44	15	19	25	33	12	15
1945–54	25	32	42	54	24	31
Confirmed only						
0–1						
1935–44	41	43	54	57	46	48
1945–54	59	62	76	80	62	65
0–5						
1935–44	21	27	35	43	14	18
1945–54	30	38	46	59	26	33
Females						
Confirmed and not confirmed, combined						
0–1						
1935–44	38	39	50	52	38	39
1945–54	57	59	76	78	63	65
0–5						
1935–44	19	23	30	37	15	17
1945–54	33	39	54	64	30	35
Confirmed only						
0–1						
1935–44	49	50	64	66	46	47
1945–54	65	67	82	84	68	70
0–5						
1935–44	28	32	42	49	21	23
1945–54	39	45	60	69	32	38

* Includes "distant" and "stage unspecified."

other treatment, and no treatment. The method of therapy for virtually all patients that were treated for cancer of the large intestine was surgery. Only 2 percent was treated by other methods (table 6). Treatment, as defined by the Connecticut Tumor Registry, consists of procedures directed against the tumor. Thus, an exploratory laparotomy would be classified "no treatment," and a palliative procedure which included resection of part of a tumor would be classified "surgery." The data at hand contain no information on the part of the large intestine affected or on the type of surgery performed.

Whereas in 1935–44 about 1 in 4 patients was untreated, in 1945–54 only 1 in 6 remained untreated. This is a very important gain, because

TABLE 6.—Percentage distribution according to treatment during first course of therapy, by sex and period of diagnosis: patients with cancer of the large intestine diagnosed in Connecticut, 1935–54

	Total number of patients	Percent			
		Total, treated and not treated	Surgery	Other treatment	Not treated
Males					
1935–44	1,167	100	69	3	28
1945–54	2,064	100	80	1	19
Females					
1935–44	1,291	100	72	2	26
1945–54	2,606	100	82	2	16

virtually none of the untreated patients lived 5 years (table 7). Also from the first period to the second, 5-year relative survival among surgically treated patients increased from 26 to 39 in men and from 30 to 46 in women. The increased use of surgical intervention combined with improvement in rate of surgical survival resulted in the very marked overall progress cited earlier. Southwick and Cole (*11*) have discussed the

TABLE 7.—Relative survival rates by treatment during first course of medical care, sex, and period of diagnosis: patients with cancer of the large intestine diagnosed in Connecticut, 1935–54*

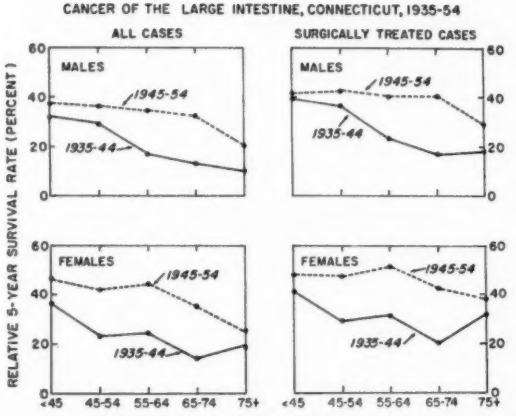
	Total, treated and not treated		Surgery		Not treated	
	Relative survival rate	Standard error	Relative survival rate	Standard error	Relative survival rate	Standard error
Males						
1935–44						
0–1 year	34	1.4	43	1.8	10	1.7
1–5 years	56	3.4	60	3.3	n.r.	—
0–5 years	19	1.4	26	1.8	2	1.0
5–10 years	87	4.5	87	4.5	n.r.	—
0–10 years	18	1.6	24	2.1	n.r.	—
1945–54						
0–1 year	55	1.2	65	1.3	12	1.7
1–5 years	58	2.4	60	2.5	*14*	7.4
0–5 years	32	1.5	39	1.8	2	1.1
Females						
1935–44						
0–1 year	39	1.4	50	1.7	11	1.7
1–5 years	59	2.6	60	2.7	*19*	8.0
0–5 years	23	1.3	30	1.7	2	0.9
5–10 years	88	3.4	89	3.4	n.r.	—
0–10 years	21	1.4	27	1.9	n.r.	—
1945–54						
0–1 year	59	1.0	69	1.1	15	1.8
1–5 years	66	1.9	67	1.9	*14*	6.8
0–5 years	39	1.3	46	1.5	2	1.0

*Rates are expressed as percents. Rates in italics have standard errors between 5.0 and 9.9. Rates with standard errors of 10.0 or larger are not shown (n.r. = not reliable).

"numerous and significant" modifications in surgical technique in cancer of the colon resulting from the use of antibiotics in the middle and late 1940's. Among these are replacement of the obstructive resection by primary anastomosis without colostomy and more adequate removal of regional nodes. We note that virtually all this improvement took place during the 1st year, which suggests that reduction in operative mortality was an important factor. Other investigators have reported large reductions in operative mortality in recent years (*12–15*).

Age

To evaluate the relationship between survival and age, we took normal survival expectancy into account. Text-figure 5 and table 8 show relative 5-year survival rates by age at diagnosis for each of 5 age groups. In Connecticut, the younger the patient with cancer of the large intestine, the more favorable the prognosis even after correction for normal mortality. The substantial improvement in rate of survival achieved during 1945–54 shows up at all ages, but is greatest in older patients, particularly in those aged 55–74. Among the surgically treated patients, the improvement in survival rate of patients aged 55–74 resulted in an approximately uniform relative rate at ages under 75 during the second period; the rate for patients 75 and over was somewhat lower.



TEXT-FIGURE 5.—Five-year relative survival rates by age at diagnosis: patients with cancer of the large intestine diagnosed in Connecticut, 1935–44 and 1945–54.

CANCER OF THE RECTUM

Age and Sex

Of the 5,082 cases of cancer of the rectum, 2,922 were in men and 2,160 were in women. As for cancer of the large intestine, we divided

TABLE 8.—Five-year survival rates by age, first course of therapy, calendar period of diagnosis, and sex: patients with cancer of the large intestine diagnosed in Connecticut, 1935–54*

Sex, therapy, and period of diagnosis	Under 45			45–54			55–64			65–74			75 and over		
	Crude rate	Relative Rate	Relative Standard error	Crude rate	Relative Rate	Relative Standard error	Crude rate	Relative Rate	Relative Standard error	Crude rate	Relative Rate	Relative Standard error	Crude rate	Relative Rate	Relative Standard error
Males															
Treated and not treated, combined															
1935–44	31	32	4.6	27	29	3.5	15	17	2.3	9	13	2.1	4	10	3.7
1945–54	*36*	*37*	5.5	34	36	3.8	30	34	2.6	24	32	2.6	11	20	3.9
Surgery															
1935–44	*38*	39	5.4	33	36	4.2	20	23	3.0	12	17	3.0	*8*	*18*	6.5
1945–54	*40*	*41*	6.1	40	42	4.3	34	40	2.9	29	40	3.1	*16*	*29*	5.6
Females															
Treated and not treated, combined															
1935–44	36	36	3.7	22	23	2.8	21	24	2.5	11	14	2.1	9	19	4.6
1945–54	45	46	3.6	41	42	3.0	41	44	2.4	28	35	2.4	15	25	3.4
Surgery															
1935–44	40	41	4.1	28	29	3.4	28	31	3.1	15	20	3.1	*16*	*32*	7.6
1945–54	48	48	3.8	45	47	3.3	47	51	2.7	34	42	2.9	22	38	4.9

*Rates are expressed as percents. Rates in italics have standard errors between 5.0 and 9.9.

the patients into two cohorts according to calendar period of diagnosis: 1935–44 (1,915 patients) and 1945–54 (3,167 patients).

There were 7 patients with cancer of the large intestine for every 5 with cancer of the rectum. Whereas the women outnumbered the men at a ratio of 1.2 to 1 in cancer of the large intestine, the men with rectal cancer outnumbered the women at 1.4 to 1. Similar sex-ratios for rectal cancer have been reported in other communities (*8*, *9*).

The age distribution of the patients with cancer of the rectum (table 9) is strikingly similar to that for patients with cancer of the large intestine (table 1). Nine out of 10 patients with rectal cancer were 45 or older. The male patients were older by about 2 years than the female patients, and the average age increased by about 2 years from 1935–44 to 1945–54.

TABLE 9.—Percentage age distribution, by sex and calendar period of diagnosis: patients with cancer of the rectum diagnosed in Connecticut, 1935–54

	Both sexes		Male		Female	
	1935–44	1945–54	1935–44	1945–54	1935–44	1945–54
Number of patients	1, 915	3, 167	1, 107	1, 815	808	1, 352
Percent:						
All ages*	100	100	100	100	100	100
Under 45	9	8	7	6	12	9
45–54	19	15	17	14	22	17
55–64	33	32	35	33	30	30
65–74	27	29	31	31	23	28
75+	12	16	11	16	13	16
Median age	62	64	63	64	60	63
Mean age	61	63	62	64	60	62

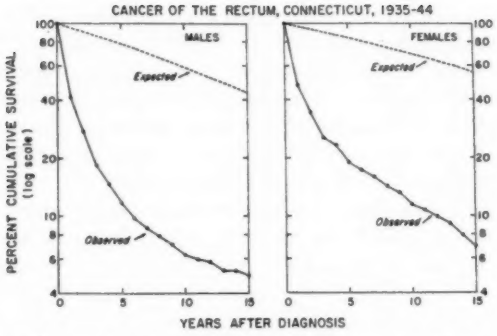
*Percentages may not total 100 because of rounding.

Survival Experience

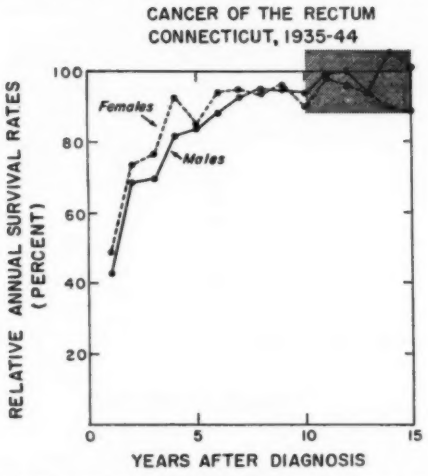
Table 10 shows that the follow-up information of the patients with cancer of the rectum is fairly complete. Only 105 patients were lost during the first 5 years of follow-up. Thirty-six of the 586 patients who were known to have survived the first 5 years were lost between the 5th and the 10th year. Of the patients diagnosed in the first period, 1935–44, 13 of the 140 (a rather large proportion) known to be alive at the beginning of the 11th year were lost before the end of the 15th year. This limits the reliability of the survival rates computed for the 10- to 15-year interval.

Text-figure 6 shows the survival experience over 15 years of follow-up of the patients with rectal cancer diagnosed during the first period, 1935–44. Percent cumulative survival is plotted on a logarithmic scale. For both males and females, the slope of the initial part of the curve is very steep, which indicates very high mortality. For comparison, we are showing the expected survival experience for a group of persons in the general population with an age distribution similar to that of the patients.

The *annual relative survival rates*, *i.e.*, the ratios of the observed to the expected annual survival rates, are shown in text-figure 7. The annual



TEXT-FIGURE 6.—Observed and expected survival rates for 15 years of follow-up: patients with cancer of the rectum diagnosed in Connecticut, 1935–44.



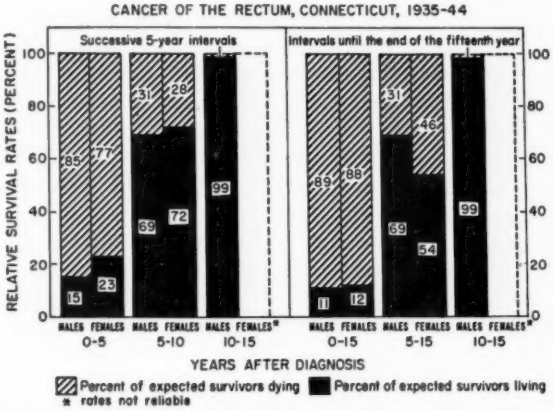
TEXT-FIGURE 7.—Relative annual survival rates for 15 years of follow-up: patients with cancer of the rectum diagnosed in Connecticut, 1935–44. (*Shaded area* indicates that the rates in this interval are based on small numbers of cases and therefore subject to large sampling variation.)

TABLE 10.—Follow-up status of patients with cancer of the rectum diagnosed in Connecticut, 1935–54, by 5-year follow-up interval and calendar period of diagnosis

Calendar period of diagnosis and follow-up interval	Alive at beginning of interval	Died during interval	Lost to follow-up during interval	Withdrawn alive during interval*
Patients diagnosed 1935–44				
0–5 years	1, 915	1, 620	23	0
5–10 years	272	112	17	3
10–15 years	140	26	13	71
Patients diagnosed 1945–54				
0–5 years	3, 167	1, 970	82	801
5–10 years	314	50	19	245

*Alive on closing date of study, December 31, 1954.

relative survival rates increased from less than 50 in the 1st year of diagnosis to about 90 in the 10th year. This means that mortality among the rectal-cancer patients was greater than normal throughout the first 10 years of follow-up. The annual relative rates between the 10th and 15th years suggest that excess mortality for male patients may have been eliminated during this interval; however, the rates are based on fairly small numbers of patients and, as was pointed out, a rather large proportion of the cases was lost to follow-up during this period; therefore the rates are not reliable.



TEXT-FIGURE 8.—Relative survival rates for specified follow-up intervals: patients with cancer of the rectum diagnosed in Connecticut, 1935–44. (Rate for females in the 10- 15-year interval is not shown because it is not statistically reliable.)

The *left panel* of text-figure 8 summarizes the information in text-figure 7 over successive 5-year intervals. Relative to expectation, only 15 percent of the males and 23 percent of the females survived the first 5 years. Of the patients alive at the end of the 5th year, only about 70 percent of those expected to survive the 5- to 10-year interval actually survived, *i.e.*, about 30 percent of the expected survivors died during the 5- to 10-year interval. Only about 5 percent of the original patient cohort survived the first 10 years, and the information is too scanty for a valid statistical analysis from the 10th year forward, but the data suggest that male survivors of the first 10 years after diagnosis with rectal cancer may achieve normal survival between the 10th and 15th year.

Comparison With Cancer of the Large Intestine

The relative 5-year survival rate for male rectal-cancer patients was somewhat lower than that for cancer of the large intestine (15 vs. 19%). The rates for females were equal for the 2 sites (23%). Among survivors of the first 5 years, patients with cancer of the large intestine fared considerably better: About 90 percent of expected survivors survived from

the 5th through the 10th year, contrasted with only 70 percent for rectal cancer. As will be shown later, the latter rate increased to 90 percent for cases diagnosed 1945–54.

Variation by Sex

Table 11 shows that during 1935–44 females had a better chance of surviving rectal cancer for 5 years, the relative rates being 23 percent for females and 15 for males. By 1945-54 most of their advantage had disappeared, *i.e.*, 5-year survival was nearly the same for men and women, about 31 percent. There was no evidence of sex difference in relative survival for the 5- to 10-year interval in either period.

It is of interest to examine the sex difference in 5-year relative survival more closely. In the earlier period of diagnosis females had a somewhat higher 1-year relative survival rate than males (49 vs. 43%), and a substantially higher relative survival rate from the 1st through the 5th year (47 vs. 35%). During the later period, females still enjoyed a somewhat better 1-year rate (63 vs. 58), but the 1- to 5-year rates were nearly identical (51 vs. 52). Thus, the only large sex difference in survival occurred during the 1- to 5-year interval for the cases diagnosed in 1935–44. An explanation for this difference, or why the difference vanished for cases diagnosed during the subsequent 10-year period of diagnosis, is not apparent to the authors. The 1- to 5-year interval after diagnosis represents the period in which the patient has survived the major risks of the operation and its after-effects, but is still liable to mortality due to metastasis.

Secular Trend

In rectal cancer we note the same dramatic improvement from 1935–44 to 1945–54 that we observed for cancer of the large intestine (table 11 and text-fig. 9). As we noted earlier, men had a lower 5-year survival in 1935–44 than women, but in 1945–54 the rates were about the same for the 2 sexes. Thus, the improvement in 5-year survival was greater for men (from 15 to 30) than for women (from 23 to 32). For men, the improvement resulted in part from an increase in 1-year survival (from 43 to 58), and in part from an increase in survival for the 1- to 5-year interval (from 35 to 58). For women, however, most of the improvement resulted from an increase in 1-year survival (from 49 to 63), the 1- to 5-year rate increasing but slightly (from 47 to 51). There was also a striking improvement—from about 70 to 90 percent—in relative survival for the 5- to 10-year interval for both men and women.

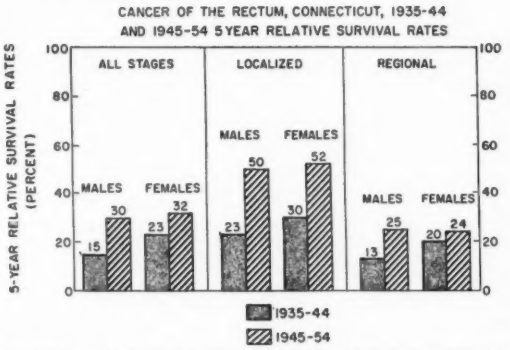
Variation by Stage of Disease

Table 12 shows the distribution of cases by stage of disease; since there was no sex difference in the stage distribution, we combined the 2 sexes. Table 13 and text-figure 9 show the survival experience by stage of disease. The pattern here is similar to that for cancer of the

TABLE 11.—Survival experience, by sex: patients with cancer of the rectum diagnosed in Connecticut, 1935–44 and 1945–54*

	Males			Females		
		Relative			Relative	
	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error
1935–44						
0–1	41	43	1.6	47	49	1.8
0–5	12	15	1.2	19	23	1.7
0–10	6	11	1.3	11	17	1.7
0–15	5	11	1.6	7	12	2.2
1–5	28	35	2.5	40	47	3.0
5–10	*54*	*69*	5.7	*60*	*78*	5.0
10–15	*78*	*99*	8.4	n.r.	n.r.	—
1945–54						
0–1	55	58	1.3	61	63	1.4
0–5	24	30	1.6	27	32	1.8
0–10	17	29	2.7	21	31	2.7
1–5	43	52	2.5	44	51	2.6
5–10	*70*	*92*	7.3	*79*	*93*	6.4

*Rates are expressed as percents. Rates in italics have standard errors between 5.0 and 9.9. Rates with standard errors of 10.0 or larger are not shown (n.r. = not reliable).



TEXT-FIGURE 9.—Comparison of 5-year relative survival rates: patients with cancer of the rectum diagnosed in Connecticut, 1935–44 vs. 1945–54.

large intestine: Five-year survival for the "localized" cases was about double that for the "regional" cases, and for patients with both localized and regional spread of disease, survival improved markedly from the first to the second period. We note, as we did for cancer of the large intestine, that the proportion of cases classified as "localized" decreased from the first to the second period. Again, this is not necessarily the result of a bona fide shift in stage composition, but may be a consequence of the relative increase in the number of patients operated on, which resulted in a greater opportunity to discover metastases.

Treatment

In 1935–44, 70 percent of the rectal cancer patients were treated by surgery, 10 percent by other methods (mostly surgery plus radiation and

TABLE 12.—Stage distribution by microscopic confirmation of diagnosis: patients with cancer of the rectum diagnosed in Connecticut, 1935–44 and 1945–54 (includes 15 patients of unknown age who were excluded from survival computations)

	All stages	Localized	Regional	Distant	Stage unknown
1935–44					
Confirmed and not confirmed, combined					
Number of patients	1,918	929	460	360	169
Percent	100	48	24	19	9
Confirmed only					
Number of patients	1,308	672	341	215	80
Percent	100	51	26	16	6
1945–54					
Confirmed and not confirmed, combined					
Number of patients	3,179	1,357	976	526	320
Percent	100	43	31	17	10
Confirmed only					
Number of patients	2,761	1,253	897	421	190
Percent	100	45	32	15	7

TABLE 13.—Crude and relative survival rates by stage of disease and microscopic confirmation of diagnosis: patients with cancer of the rectum diagnosed in Connecticut, 1935–44 and 1945–54

Sex, confirmation, follow-up interval in years, and period of diagnosis	All stages*		Localized		Regional	
	Crude survival rate	Relative survival rate	Crude survival rate	Relative survival rate	Crude survival rate	Relative survival rate
Males						
Confirmed and not confirmed, combined						
0–1						
1935–44	41	43	50	52	43	45
1945–54	55	58	70	73	56	59
0–5						
1935–44	12	15	18	23	10	13
1945–54	24	30	40	50	20	25
Confirmed only						
0–1						
1935–44	47	49	56	58	46	48
1945–54	60	63	74	77	60	62
0–5						
1935–44	16	21	24	29	14	17
1945–54	27	34	43	54	22	27
Females						
Confirmed and not confirmed, combined						
0–1						
1935–44	47	49	56	58	50	52
1945–54	61	63	77	79	59	61
0–5						
1935–44	19	23	25	30	17	20
1945–54	27	32	44	52	21	24
Confirmed only						
0–1						
1935–44	57	58	68	70	58	60
1945–54	66	68	80	83	63	65
0–5						
1935–44	26	30	35	41	22	26
1945–54	30	35	48	55	22	25

*Includes "distant" and "stage unspecified."

TABLE 14.—Percentage distribution according to treatment during first course of therapy, by sex and period of diagnosis: patients with cancer of the rectum diagnosed in Connecticut, 1935–54

	Total number of patients	Percent			
		Total, treated and not treated	Surgery	Other treatment	Not treated
Males					
1935–44	1,107	100	69	11	20
1945–54	1,815	100	81	3	16
Females					
1935–44	808	100	71	9	20
1945–54	1,352	100	83	4	13

radiation only), and 20 percent were untreated (table 14). By 1945–54, the percent treated by surgery alone increased to 82 percent, other treatments decreased to 3 percent, and untreated to 15 percent.

Survival among the surgically treated patients increased significantly for both men and women from the first to the second period (table 15). In men, the 5-year relative rate increased from 20 to 36, and in women from 29 to 37. It is interesting that, whereas the outlook after surgery was more favorable for women in the first period, this sex difference disappeared in the second period. In men, the increase is a product of

TABLE 15.—Relative survival rates by treatment during first course of medical care, sex, and period of diagnosis: patients with cancer of the rectum diagnosed in Connecticut, 1935–54*

	Total, treated and not treated		Surgery		Not treated	
	Relative survival rate	Standard error	Relative survival rate	Standard error	Relative survival rate	Standard error
Males						
1935–44						
0–1 year	43	1.6	48	1.9	23	2.9
1–5 years	35	2.5	42	3.2	*9*	5.3
0–5 years	15	1.2	20	1.7	2	1.2
5–10 years	*69*	5.7	*71*	5.8	0	—
0–10 years	11	1.3	14	1.7	0	—
1945–54						
0–1 year	58	1.3	65	1.4	23	2.6
1–5 years	52	2.5	55	2.5	*20*	7.7
0–5 years	30	1.6	36	1.8	5	2.0
Females						
1935–44						
0–1 year	49	1.8	56	2.1	17	3.1
1–5 years	47	3.0	52	3.2	*13*	8.8
0–5 years	23	1.7	29	2.1	2	1.4
5–10 years	*78*	5.0	*75*	5.0	0	—
0–10 years	17	1.7	22	2.2	0	—
1945–54						
0–1 year	63	1.4	69	1.5	23	3.3
1–5 years	51	2.6	54	2.7	*18*	9.6
0–5 years	32	1.8	37	2.0	4	2.2

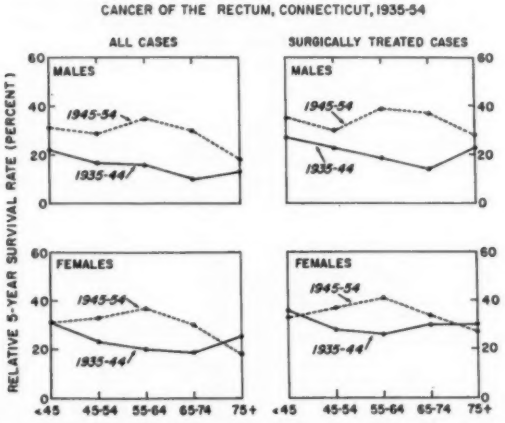
*Rates are expressed as percents. Rates in italics have standard errors between 5.0 and 9.9.

improvement in both the 0 to 1- and 1 to 5-year rates, but in women almost all the improvement resulted from an increase in 0 to 1 year survival. Survival in the untreated group was virtually nil. Thus, the outstanding factors in the improvement of end results in cancer of the rectum are the same that we noted for cancer of the large intestine: increased use of surgery and improvement in survival of surgically treated patients. The proportion of patients not treated in 1945–54 was the same for large intestine and rectum, about one sixth.

Ottenheimer reviewed the Connecticut experience for cancer of the rectum for 1935–45 (*16*). This was the first survival analysis attempted with data from the Connecticut Tumor Registry. He found a sharp decrease in the use of the two-stage resection and a corresponding increase in the one-stage resection. This resulted in a decrease in operative mortality from 25 percent in 1935–40 to 18 percent in 1941–45. It is noteworthy that the decline in operative mortality began before the widespread introduction of antibiotics.

Age

Text-figure 10 and table 16 show the relative 5-year survival rates by age at diagnosis for each of 5 age groups. Some of the rates, particularly for the patients under 45 and 75 or older, are, as indicated by the large standard errors, based on a small number of cases.



TEXT-FIGURE 10.—Five-year relative survival rates by age at diagnosis: patients with cancer of the rectum diagnosed in Connecticut, 1935–44 and 1945–54.

In 1935–44, 5-year relative survival decreased progressively with age for patients under 75, and then increased slightly for patients 75+. By 1945–54, the age-survival pattern had changed: Patients aged 55 to 64 experienced peak survival.

TABLE 16.—Five-year survival rate by age, first course of therapy, calendar period of diagnosis, and sex: patients with cancer of the rectum diagnosed in Connecticut, 1935–54*

Sex, therapy, and period of diagnosis	Under 45			45–54			55–64			65–74			75 and over		
		Relative			Relative			Relative			Relative			Relative	
	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error	Crude rate	Rate	Standard error
Males															
Treated and not treated, combined															
1935–44	22	22	4.9	16	17	3.0	13	16	2.1	7	10	2.0	6	13	4.7
1945–54	*31*	*31*	5.5	27	29	3.8	30	35	2.6	22	30	3.0	10	18	3.9
Surgery															
1935–44	26	*27*	6.0	21	23	3.8	16	19	2.6	10	14	2.8	*10*	*23*	9.0
1945–54	*35*	*35*	6.1	29	30	4.1	34	39	2.9	27	37	3.7	*16*	*28*	6.0
Females															
Treated and not treated, combined															
1935–44	30	31	4.7	22	23	3.3	18	20	2.8	15	19	3.4	*12*	*25*	6.5
1945–54	30	31	4.9	32	33	3.8	34	37	3.1	24	30	3.5	*10*	*18*	5.2
Surgery															
1935–44	*35*	*36*	5.4	26	28	4.0	24	26	3.5	*23*	*30*	5.2	*15*	*30*	9.3
1945–54	*32*	*33*	5.2	36	37	4.3	38	41	3.4	27	34	4.0	*16*	*27*	7.9

*Rates are expressed as percents. Rates in italics have standard errors between 5.0 and 9.9.

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Occurrence of Visceral Lymphomatosis in Chickens Inoculated With Rous Sarcoma Virus[1]

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SUMMARY

In 3 experiments involving 760 line 15I chickens, inoculation with high dilutions of a standard preparation Rous sarcoma virus by the intravenous route caused not only Rous sarcoma but also a significant incidence of visceral lymphomatosis and erythroblastosis during the 70 to 270 days after inoculation. Rous sarcoma and visceral lymphomatosis also occurred in contact chickens, but none of the total of 99 chickens of the same source and hatches that were raised in cubicles by themselves developed any neoplasm.—J. Nat. Cancer Inst. 26: 511–518, 1961.

VISCERAL LYMPHOMATOSIS, also known as lymphoid leukosis or lymphocytoma, occurs naturally in most flocks of domestic chickens. It has been regularly reproduced by viral filtrates of not only lymphoid tumor but of plasma, oral washings, fecal extracts, and embryonating eggs (*1–4*). Virus from all these sources, in addition to causing visceral lymphomatosis, has also, with one exception, caused erythroblastosis, and virus from a few sources has caused also osteopetrosis and/or other neoplasms (*5*). Of particular interest was the finding that 2 leukosis strains, which were extensively studied by Dr. J. W. Beard and coworkers and considered "pure" on the basis of their studies, have been found to cause more than one neoplasm when the inoculated chickens were observed for long periods. Strain R brought about visceral lymphomatosis in addition to erythroblastosis, and BAI strain A induced visceral lymphomatosis, renal adenocarcinoma, and osteopetrosis, in addition to myeloblastosis (*6*).

Association between fibrosarcomas and leukosis, especially erythroblastosis, has been the subject of much study. At least 5 strains have been described that cause both erythroblastosis and sarcomas by the same route of inoculation (*7–11*). Several investigators (*12–15*) thought that both neoplasms were caused by one virus and that such factors as route and host variation were important in the determination of the response.

[1] Received for publication September 29, 1960.

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Others considered such strains to be a mixture of 2 or more viruses. Pikovski (*16*) showed that the type of inoculum is also an important factor. He obtained only leukosis when plasma or cell-free liver extracts of the "pure" erythroblastosis strain T was given intramuscularly, but when whole blood or liver suspensions of the same source were inoculated by the same route, leukosis and fibrosarcomas developed.

Duran-Reynals *et al.* (*17*) reported that chickens reared in isolation and showing little or no lymphomatosis were devoid of antibodies that neutralize Rous sarcoma virus, but that when the same strain of chickens was exposed and developed a high incidence of lymphomatosis, the serums from most of these hens had Rous virus neutralizing antibodies. A close antigenic relationship between the visceral lymphomatosis and Rous sarcoma viruses was suggested. Similar data on the natural occurrence of Rous sarcoma virus antibodies and mortality from lymphomatosis were obtained by Kenzy (*18–20*).

Reciprocal cross-neutralization between the virus and the antibodies of Rous sarcoma and those of strain RPL12 erythroblastosis-visceral lymphomatosis has been reported (*21*) and adds further weight to the evidence of a close antigenic relationship between the viruses causing fibrosarcomas and those causing the various leukoses.

It should be emphasized that such evidence is not above question, since proof of the use of uncontaminated reagents is lacking. Common occurrence of inapparent infections with visceral lymphomatosis virus has been adequately demonstrated (*4, 22*), and the occurrence of such types of infections with the fibrosarcoma viruses has also been suggested (*23–26*).

Kenzy (*27*) reported the occurrence of visceral lymphomatosis in a high proportion of the birds that had been inoculated with suspensions and a filtrate of two myxosarcomatous tumors that occurred naturally in 2 different flocks of New Hampshire Red pullets. Of a total of 66 birds inoculated, only 2 developed connective-tissue tumors, whereas 45 died of visceral lymphomatosis. Five possibilities were offered to explain the phenomenon observed. The most probable explanation is that, even though the 2 donor birds showed no evidence of visceral lymphomatosis, there was much of this disease in the flock or in an adjoining flock on the same farm. Thus there existed the prerequisite conditions for much inapparent infections, and the tumors used may well have contained large amounts of visceral lymphomatosis virus.

Since the first isolation of the Rous sarcoma virus in 1911 (*28*), extensive investigations concerning all phases of the virus-host relationships and the host responses have been conducted in numerous laboratories and with many different breeds and strains of chickens.

Throughout this history of experimentation, the virus has remained consistent, especially with regard to its pathologic manifestations. Duran-Reynals (*29, 30*) obtained variants by the inoculation of young ducks, turkeys, and guinea fowls. These virus variants in chickens caused tumors that were usually collagenous and were often located in the periosteum and endosteum. In addition, a low incidence of osteopetrosis, 1 myeloid

tumor, and 3 (2%) lymphoid tumors of the ovary were observed. He considered these tumors to be due to the variant virus. Whether or not the low incidence of lymphoid tumors observed was due to the variant virus is questionable, since such tumors commonly occur and inapparent infection is naturally present in most stocks of chickens.

In view of the results obtained with the "pure" leukosis strains R and A, it was of unusual interest to investigate the types of neoplasms that could be obtained in line 15I chickens inoculated with a standard Rous sarcoma virus preparation given at very low doses so that the response period was prolonged. The results of 3 experiments are reported here. Data relative to contact transmission of Rous sarcoma obtained in 2 of the experiments have been reported elsewhere (*31*).

MATERIALS AND METHODS

The virus was a part of a standard virus pool identified as CT750 and prepared at the National Cancer Institute by methods described (*32*). Aliquots in volumes of 0.15 ml had been sealed in small ampules, frozen, and stored in a dry-ice chest. A sufficient number of ampules of virus for these as well as other experiments was obtained through the courtesy of Dr. W. Ray Bryan. Dilutions were made with 0.85 percent NaCl solution to which was added 2 percent normal horse serum.

White Leghorns of inbred line 15I that have been found quite susceptible to the development of visceral lymphomatosis and other neoplasms (*33*) were inoculated at 9 days of age by the intravenous (cubital vein) route with 0.2 ml of inoculum. All chickens were raised in cubicles (*2*) until about 11 weeks of age, after which they were transferred to large pens. Each lot was placed in a separate cubicle, except for those groups not inoculated and considered "contacts." These chickens were raised in the same cubicle and allowed to intermingle with inoculated birds of the same hatch. Other groups of noninoculated chickens placed alone in cubicles were considered controls. They were allowed to mix with inoculated birds after they were moved to large pens. The experimental period for all groups was 270 days. Examinations and the basis for the diagnosis were the same as those previously described (*5*).

RESULTS

Data of the 3 experiments showing mortality from visceral lymphomatosis and erythroblastosis, as well as mortality from Rous sarcoma after inoculation with a preparation from Rous sarcoma, are given in table 1. The percentage data are based on the inoculated number corrected for those that died prior to the age period wherein specific deaths from the neoplasm in question almost invariably occurred. For a more detailed discussion *see* previous reports (*5*, *6*).

TABLE 1.—Mortality from Rous sarcoma, visceral lymphomatosis, and erythroblastosis in chickens inoculated or in contact with Rous sarcoma virus

Treatment	Dilution (logs)	Numbers inoculated				Mortality from neoplasms					
			Corrected* for			Number			Percent		
		Total	Rous sarcoma	Erythro-blastosis	Visceral lympho-matosis	Rous sarcoma	Erythro-blastosis	Visceral lympho-matosis	Rous sarcoma	Erythro-blastosis	Visceral lympho-matosis
Experiment 1											
Inoculated	−6. 3	10	9	5†	4	4	0	1	44. 5	0	25. 0
Inoculated	−7. 3	10	10†	10†	7	0	0	2	0	0	28. 5
Inoculated	−8. 3	10	10†	8†	6	0	0	2	0	0	33. 3
Experiment 2											
Inoculated	−5. 3	120	118	8†	5	108	0	0	91. 6	0	0
Inoculated	−5. 8	80	79	8†	6	70	0	1	88. 6	0	16. 6
Inoculated	−6. 3	60	60	17	13	39	3	7	65. 0	17. 6	53. 9
Contacts with −5. 3	—	60	59	55†	46	6	0	10	10. 2	0	21. 7
Controls	—	50	49	49†	45	0	0	0	0	0	0
Experiment 3											
Inoculated	−6. 3	180	178	76	64	99	3	14	55. 6	4. 0	21. 9
Inoculated	−6. 8	70	66	64†	50	13	0	14	19. 7	0	28. 0
Contacts with −6. 3	—	60	60	59†	50	3	0	2	5. 0	0	4. 0
Controls	—	50	50†	50†	44	0	0	0	0	0	0

*See text.
†Estimated.

The first experiment was exploratory. Six lots of 10 birds each were inoculated with several log dilutions of virus ranging from 3.2 to 8.3. Only the 3 highest dilutions resulted in some survivors beyond the period of Rous sarcoma mortality. Of the 17 birds that survived this period, 5 developed visceral lymphomatosis. Although the numbers were small, the results were of sufficient interest for additional experiments.

Almost all chickens of experiment 2 that were inoculated with a −5.3 log dilution died of Rous sarcoma, and none were left to develop visceral lymphomatosis. However, of 60 noninoculated chickens in direct contact with the inoculated chickens, 6 developed Rous sarcomas and 10 others died of visceral lymphomatosis. Other lots were inoculated with −5.8 or −6.3 logs dilution and of 23 survivors 8 developed visceral lymphomatosis and 3 erythroblastosis. None of 49 controls developed a neoplasm.

In a subsequent experiment, 2 lots of chickens were inoculated with −6.3 or −6.8 logs dilution, and of a total of 126 survivors 28 developed visceral lymphomatosis. As in the previous experiment, 3 birds inoculated with the −6.3 logs dilution of virus died of erythroblastosis. Of 60 chickens raised in contact with the latter inoculated group, 3 developed sarcomas and 2 developed visceral lymphomatosis. Again, all 50 controls were negative. In the 3 experiments, the time from inoculation to death from visceral lymphomatosis ranged from 124 to 270 days and from erythroblastosis 70 to 227 days.

The high mortality resulting from Rous sarcomas, induced by the inoculation of moderate-to-high dilutions of the CT750 preparation by the intravenous route, indicates that this route is highly sensitive to Rous virus of this preparation. Chickens of the same source were also inoculated by the subcutaneous and the intracranial routes. On the basis of the LD50's derived from probit plots, equivalent responses were obtained at −6.5, −5.5, and −5.5 log dilutions of virus for the intravenous, subcutaneous, and intracranial routes, respectively.

DISCUSSION

Results obtained in 3 experiments show that the Rous sarcoma virus preparation, CT750, when inoculated intravenously, caused visceral lymphomatosis and erythroblastosis, in addition to Rous sarcomas. Thus, of a total of 262 chickens that lived beyond the period of death due to Rous sarcoma, 53 died of visceral lymphomatosis and 6 of erythroblastosis. None of 99 control birds developed any neoplasms during the same 270-day experimental period. The lack of any tumors in the controls demonstrated that the chickens were not naturally infected nor did they acquire the infection from the environment, at least at a level sufficiently high to induce tumors. Since the leukotic diseases usually require a longer time-to-death than Rous sarcomas, doses of Rous virus had to be sufficiently low so that an appreciable number of birds survived the period wherein Rous sarcomas caused mortality. Thus, at a dilution of −6.8

logs, only 20 percent developed Rous sarcoma and 28 percent of the survivors died of visceral lymphomatosis. At dilutions beyond this, none died of Rous sarcomas, but 4 of 13 died of visceral lymphomatosis (expt. 1).

This demonstration of the induction of visceral lymphomatosis and erythroblastosis by a standard preparation of Rous sarcoma virus may largely be attributed to the combination of the following 3 factors: 1) the use of chickens that are highly susceptible to the development of visceral lymphomatosis and, at the same time, have a very low level of natural infection, 2) the employment of low doses of virus, and 3) the maintenance of the chickens for a sufficiently long period to permit the development of visceral lymphomatosis. Whether or not the use of the intravenous route of inoculation, which proved to be as efficacious as the subcutaneous route in causing death from sarcomas, was a significant factor in the causation of leukosis cannot be determined from the data.

These results again prompt the question of whether the several neoplasms obtained were caused by a single, multipotent virus or whether each was induced by a separate, specific virus. These and other available data do not provide the critical evidence required to answer this question.

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A Simple Method for the Preparation of Potent Chicken Anti-Rous Sarcoma Virus Serum [1]

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SUMMARY

A method is described for the immunization of chickens with either formalin or heat-killed Rous sarcoma virus incorporated in Freund's complete adjuvant. Serums from immunized birds were titrated for neutralizing antibody in chick embryos prior to and after a subsequent injection of viable virus. More than half the birds, all of which had a high antibody content after vaccination, survived the challenge inoculation, and a high concentration of antibody persisted up to a year.—J. Nat. Cancer Inst. 26: 519–522, 1961.

THE DIFFICULTY of obtaining a satisfactory antiserum for immunological studies with Rous sarcoma virus (RSV) has been known for some time (*1*). The availability of chicken anti-RSV serum has been dependent upon the fortuitous occurrence of birds in which a tumor invoked by the inoculation of viable RSV has regressed. A simple method for the production of high-titered chicken anti-RSV serum, utilizing the principle of increasing the antibody response to killed virus by incorporating it into Freund's adjuvant, is described herein.

MATERIALS AND METHODS

Chickens.—New Hampshire Red chickens 2 months of age and of both sexes were used.

Virus.—A strain of RSV of high infectivity titer (*2*) was used throughout. It was prepared by the method used routinely in this laboratory (*3*). Prior to immunization, the virus was treated in one of two ways: 1) Formalin (HCHO) was added to a final concentration of 0.1 percent and the preparation refrigerated at 5° C for 19 days; or 2) the virus suspension was heated at 65° C in a water bath for 30 minutes, then allowed to remain in the refrigerator for 5 days. On the day of injection a portion of each

[1] Received for publication October 25, 1960.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

preparation of virus was emulsified with an equal portion of Freund's complete adjuvant.[3]

Immunization of chickens.—Three birds were inoculated with formalin-treated virus; four with heated virus. A total of 2 ml of virus-adjuvant mixture was injected into each chicken, with 0.5 ml administered intramuscularly into each of four sites in the breast. The birds were bled from the heart 26 days after vaccination, and the serum separated and stored at −70° C. The chickens were then inoculated subcutaneously in the left wing web with 0.2 ml of a 10^{-2} dilution of standard virus and observed for the development of tumors.

At certain intervals after the injection of the viable virus, the birds were bled and the serums stored individually. Chickens that survived the challenge inoculum were bled at intervals for several months and serum pools prepared, representing approximately equivalent amounts of serum from each bird. Four such pools were prepared, with a booster of 0.35 ml containing approximately 10^6 ED50 of heat-killed virus injected subcutaneously 1 week before the last bleeding.

Titration of neutralizing antibody content of the serum.—After all the serums were collected, the neutralization index (NI) of each was determined with a pock-counting method in chick embryos for routine determination of antibody levels in large numbers of serums (*4*). An equal volume of one or more dilutions of inactivated serum was mixed with serial tenfold dilutions of standard RSV and incubated at room temperature for 30 minutes. Afterwards, 0.1 ml amounts of each mixture were inoculated onto the chorio-allantoic membrane of six to nine 9-day-old embryonated hen's eggs. Control mixtures similarly inoculated included: 1) diluent and appropriate dilutions of RSV, 2) a known positive serum plus RSV, and 3) a known negative serum plus RSV. The NI was determined by subtraction of the log of the average number of pock-forming units per ml in eggs inoculated with virus-antiserum mixtures from that produced with the virus diluent alone. When the diluted serum neutralized all of the most concentrated virus, the NI was expressed as being greater than the log number of pock-forming units contained in that mixture.

RESULTS

The results expressed in table 1 indicate that vaccination with either heat or formalin-inactivated RSV incorporated in Freund's complete adjuvant will stimulate sufficient antibody to enable over half the birds to withstand challenge with a high dose of viable virus.

All those birds that reacted to the challenge by the production of tumors which subsequently regressed had a neutralization index within a range of 2.41 to 3.97, while those which were killed when all hope of regression of the tumor was passed had NI's ranging from 0.66 to 1.43.

[3] Composed of 1 part of Ariacel A, Atlas Powder Co., Wilmington, Del., and 4 parts of Bayol F, Esso Standard Oil Co., New York, N.Y., containing 3 mg per ml of heat-killed, dried tubercle bacilli.

TABLE 1.—Antibody response of chickens to vaccination and subsequent challenge with viable RSV

Chicken No.	Vaccinated with	Neutralization index* (log)	Reaction to challenge†	Neutralization index after challenge (log)
1	Heated RSV	1. 43	Killed with 4+ tumor 1 month	>3.21 at death
2	Heated RSV	2. 41	4+ Tumor; regression	2.79 6 weeks later
3	Heated RSV	2. 51	1+ Tumor; regression	>3.69 3 months later
4	Heated RSV	0. 66	Killed with 4+ tumor 16 days	—
5	HCHO-RSV	2. 89	4+ Tumor; regression	>3.21 6 weeks later
6	HCHO-RSV	0. 82	Killed with 4+ tumor 1 month	0.70 at death
7	HCHO-RSV	>3. 97	4+ Tumor; regression	2.87 6 weeks later >3.69 4½ months later

*Birds were bled on 26th day after vaccination, then challenged with 0.2 ml of 10^{-2} dilution of standard RSV (approximately 10^4 ED50) injected subcutaneously in left wing web.

†Arbitrary designation of tumor, which involves ¾ or more of the wing web, is 4+; 1+ is a barely discernible reaction to the inoculum.

The four surviving birds were kept, the serums from bleedings made at different intervals were pooled, and the NI's determined. The results of these tests are recorded in table 2. An examination of this table reveals the persistence of a high titer of neutralizing antibody for as long as a year after the original vaccination. The increase in titer between the C and D pools suggests the effectiveness of a booster injection of heat-killed virus 1 week prior to the D bleeding.

TABLE 2.—Persistence of high concentration of neutralizing antibody in serum of chickens immunized with nonviable RSV in adjuvant

Experiment No.	PFU* in test mixture (log)	Final serum dilution	Log neutralization index of serum†					
			Pool A	Pool B	Pool C	Pool D	Positive control	Negative control
1	4. 41	1:10	>4. 41	>4. 41	>4. 41	>4. 41	2. 62	0. 14
2	4. 32	1:10	>4. 32	>4. 32	>4. 32	>4. 32	2. 80	0. 09
	4. 32	1:100	2. 22	3. 02	3. 32	3. 69	1. 72	0. 02
	2. 32	1:1000	0. 29	0. 21	0. 79	1. 14	0. 63	0. 00
3	4. 97	1:100	—	2. 86	2. 95	—	1. 46	—

*Pock-forming units/ml.

†Pool A represents 4 surviving chickens bled 7 to 9 months after vaccination; B: 4, one month after A; C: 3, one month after B; D: 2, one month after C and 1 week after booster injections.

CONCLUSIONS

A simple technique has been applied to the production of chicken anti-RSV serum which yields high titers of neutralizing antibody. The persistence of a high level of circulating antibody for at least a year would make it feasible for any laboratory to initiate and to maintain a relatively constant source of this antiserum.

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Announcements

Manuscripts approved for JNCI, December 15, 1960 to January 15, 1961

"Results of the French study on the role of tobacco, and specially of inhalation, in the different sites of cancer," by Daniel Schwartz, Robert Flamant, Joseph Lellough, and Pierre F. Denoix.

"Sarcomatous change and maintenance of the mammary-tumor agent in long-term cultures of mouse mammary carcinoma," by Katherine K. Sanford, Howard B. Andervont, Thelma B. Dunn, Benton B. Westfall, Lartius T. Dupree, Anne B. Covalesky, Gwendolyn L. Hobbs, and Wilton R. Earle.

"Liver enzymes during hepatic carcinogenesis," by Eberhard G. Trams, Joseph K. Inscoe, and Robert A. Resnik.

"Influence of the A^v gene on mammary-gland tumors, hepatomas, and normal growth in mice," by W. E. Heston and George Vlahakis.

"Mechanism of experimental tumorigenesis. Ultrastructural alterations in mouse epidermis caused by carcinogen administered *per os*," by Kai Setälä, Eino Erkki Niskanen, Lauri Merenmies, Mauri Nyholm, and Leo Stjernvall.

"Alterations *in vitro* within a clone of mouse tumor cells," by K. K. Sanford, B. B. Westfall, E. Chu, E. L. Kuff, A. B. Covalesky, L. T. Dupree, G. L. Hobbs, and W. R. Earle.

"Induction of liver-cell carcinomas in hamsters with carbon tetrachloride," by Guiseppe Della Porta, Benedetto Terracini, and Philippe Shubik.

"Morphological evidence for the association of virus particles with the pancreatic acinar cells of the chick," by Robert F. Zeigel.

U.S. GOVERNMENT PRINTING OFFICE: 1961